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TITLE: Administration of Additional Phosphorylated Prolactin During Pregnancy Inhibits Mammary Ductal Branching and Promotes Premature Lobuloalveolus Development

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<b>13. ABSTRACT (Maximum 200 Words)</b>  Prolactin (PRL) is a hormone recognized as promoting both proliferation and differentiation in the mammary gland. Current theory proposes that it is the coexisting steroid environment that dictates which of these two activities predominates. During the tenure of this grant, however, we have determined that the ratio of unmodified PRL (U-PRL) to phosphorylated PRL (as judged by the use of a molecular mimic, S179D PRL) is also important in this regard. Working directly on the mammary gland, U-PRL promotes proliferation, whereas S179D PRL inhibits proliferation and yet promotes differentiation. S179D PRL also blocks estrogen-induced proliferation and progesterone-induced ductal branching and can reduce susceptibility to carcinogen-induced tumors. U-PRL and S179D PRL primarily use different signaling pathways. S179D PRL both inhibits signaling from U-PRL as well as promoting signaling to cell cycle inhibition and the expression of tissue-specific genes. These results contribute to our understanding of normal mammary biology and suggest that changes in the ratio of U-PRL to P-PRL to which the gland is exposed would make it more or less susceptible to cancerous change. In addition, an agent, like S179D PRL, which inhibits proliferation and promotes differentiation has the potential to be an important therapeutic for the prevention or treatment of breast cancer.				
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## INTRODUCTION

Prolactin (PRL) is released from the pituitary as a variety of posttranslationally modified forms (1). Work from this laboratory has concentrated on the interactive biology of the unmodified hormone and its phosphorylated counterpart, primarily because of the antagonistic activities of these two PRLs in many tissues (2-23). From the results of our studies thus far, we have developed the working hypothesis that phosphorylated PRL (P-PRL) antagonizes the growth-promoting activities of unmodified PRL (U-PRL), but that when the function of PRL in a tissue is not related to growth, the two PRLs show different degrees of the same activity. Standard PRL preparations, distributed by the NIDDK, contain a mixture of U-PRL and P-PRL and therefore produce effects which are an aggregate of the two activities. Where growth is concerned, the NIDDK standard PRL would be expected to be less efficacious than U-PRL. Where induction of a tissue-specific protein is concerned, the P-PRL would be expected to be more efficacious than the NIDDK standard PRL. In order to study the differential biology of these two PRLs, we have developed recombinant versions of each (15). The production of PRLs without posttranslational modifications was accomplished by expression in *E. coli*. The production of recombinant P-PRL was further accomplished by synthesizing a molecular mimic. This molecular mimic substitutes an aspartate residue for the normally phosphorylated serine (to produce S179D PRL). An aspartate residue mimics a phosphoserine both by size and charge of the side-chain. The advantage of the recombinant forms is that 1) they can be produced in sufficient quantities for *in vivo* studies; 2) they are completely free of contamination by each other; and 3) the molecular mimic of P-PRL cannot be dephosphorylated during experimental procedures. In addition, we have developed human versions of these two PRLs in order to be able to follow the effect of their administration on endogenous PRL production in experimental animals and so that we gain information about a molecule that could potentially be used to fight breast cancer in the human population. We have proposed, and our data now further support, that it is a balance between the proliferative and anti-apoptotic effects of U-PRL and the anti-proliferative and differentiative effects of P-PRL that controls the end-effects of total PRL. In the mammary gland, changes in the ratios of the two PRLs would result in cyclic changes in the breast, but the proliferative actions of U-PRL would be most exaggerated during pregnancy, while the differentiative actions of P-PRL would be most exaggerated during lactation. We hypothesized that excess U-PRL might result in inappropriate growth, while excess P-PRL (or administration of the molecular mimic thereof) would be protective against inappropriate growth. The purpose of this project was to determine whether our prediction of the differential roles of these two PRLs in the mammary gland held true, whether these effects were independent of steroid influences on the mammary gland and, whether treatment with the molecular mimic of P-PRL would result in refractoriness to carcinogen-induced tumors.

## BODY

### Statement of work item (1).

As detailed in the paper published in Cell and Tissue Research (see appendix published paper # 1), we have determined that U-PRL and P-PRL (the latter as mimicked by S179D PRL) have very different activities in both the pregnant and non-pregnant mammary gland. Thus, we have determined that U-PRL promotes ductal growth and branching while S179D PRL inhibits ductal branching, inhibits the growth of alveoli, while at the same time promoting  $\beta$ -casein gene

expression. Figure 1 in the appended manuscript shows equivalent sections of mammary glands from untreated term pregnant dams (panels A & D), those treated with U-PRL (B and E) and those treated with S179D PRL (C and F). Treatment with U-PRL resulted in larger lobuloalveoli than those of the control pregnant animals, whereas treatment with S179D PRL resulted in smaller lobuloalveoli than the controls. The area occupied by alveoli *versus* stroma and ducts was significantly different from controls in both treatment groups (Table 1 in manuscript). Of particular note was the finding that, in the 40% smaller glands of the S179D PRL-treated animals, the area occupied by lobules was reduced to almost half ( $p < 0.001$ ). A difference in morphological appearance of the milk/colostrum was also evident in the different treatment groups. Thus, U-PRL treatment increased the number of lipid droplets, whereas S179D PRL treatment decreased the lipid content.

Table 2 in the attached paper shows that progesterone (P), estradiol (E) and corticosterone (C) levels are unaltered by the treatments. Northern analysis of  $\beta$ -casein expression in these animals is shown in Figure 2. U-PRL treatment caused reduced  $\beta$ -casein expression, whereas S179D PRL enhanced  $\beta$ -casein expression. The ability of S179D PRL to enhance  $\beta$ -casein expression was confirmed in a rodent mammary cell line *in vitro* and this is illustrated in Figure 3.

When non-pregnant animals were exposed to the different PRLs, both resulted in mammary development by comparison to the controls. However, U-PRL did this by promoting ductal and alveolar growth, while S179D PRL by contrast, produced smaller alveoli arising from smaller ducts (Figure 4 in appended manuscript). The overall picture is best illustrated in the whole mount images shown as Figure 5. Morphometric analysis showed the ducts of the U-PRL-treated glands to be 1.4 fold the diameter of the ducts in the S179D PRL-treated and control glands (Table 3). This increase in size of the ducts was not associated with an increase in the duct's dense stroma (Table 3). P, E and C were not significantly different among the 3 groups of non-pregnant animals.

These results show a likely direct effect of the different PRLs because P, E and C were unaltered by treatment. An effect via placental products can also be eliminated since similar changes occurred in the non-pregnant animals. Thus U-PRL promotes mammary growth, while S179D PRL inhibits growth and yet promotes a differentiated function.

Statement of work item 1 is complete.

#### **Statement of work item (2).**

Part of our hypothesis is that exposure to P-PRL during late pregnancy and early lactation is responsible for the refractoriness to carcinogen-induced tumor formation seen in parous animals and women with an early first pregnancy. To test this hypothesis, we compared the incidence and time to tumor formation among four groups of 24 rats each. The first group had been pregnant, the second group had been pregnant with continual exposure to extra U-PRL throughout their pregnancy and for 1 week thereafter, the third group had been pregnant with continual exposure to extra P-PRL, in the form of S179D PRL for the same period of time, and the fourth group were virgin animals purchased, shipped and housed with the others. All animals had Alzet minipumps inserted subcutaneously. The dosage of the PRLs gave a circulating concentration of 50 ng/ml and had no effect on the concentration of P, E or C. Since the S179D

PRL-treated animals fail to lactate and each group needed to be treated equivalently, all pups were removed within 12h of birth. The carcinogen, N-methyl-nitroso urea (NMU) was administered (50 mg/kg IP) 7 days after delivery (i.e., when the pumps were empty). Based on the work of others, we anticipated that there would be more tumors in the virgin animals than in those that had gone through a pregnancy. Further, we hypothesized that administration of the extra U-PRL would reverse the pregnancy protection and second that pregnancy protection would be exaggerated by S179D PRL. This first experiment failed to reproduce the findings of others inasmuch as the animals that had been through a pregnancy had a higher incidence of tumors than the virgin animals:

4 <sup>th</sup> Month	5 <sup>th</sup> Month	6 <sup>th</sup> Month	7 <sup>th</sup> Month	8 <sup>th</sup> Month	9 <sup>th</sup> Month	10 <sup>th</sup> Month
1-12	1-12	1-12	1-12	1-12	1-12	1-12 (5X8 mm)
			1-16	1-16	1-16	1-16 (barely palpable)
1-19	1-19*	1-19	1-19	1-19	1-19	1-19 (Killed at month 5)
			1-26**	1-26	1-26	1-26 (Killed at month 7)
4-16	4-16	4-16	4-16	4-16	4-16	3-23 (43.29X32.04 mm)
						3-24 (10.72/X0.45 mm)
						4-16 (27.56/X0.21 mm)
						4-20 (33.56X24.83 mm)

Group 1, Pregnant with Saline in pump

Group 2, Pregnant with U-PRL in pump (6 $\mu$ g/24 h)

Group 3, Pregnant with S179D PRL in pump (6  $\mu$ g/24h)

Group 4, virgin group with Saline in pump

\*Killed (66.54X52.35mm, 91.29 g), \*\*Killed (32.4X30, 29.64 g)

*Table of tumor incidence and size in the treatment groups. The animal numbers are given e.g. 1-12 is an animal in group 1 which is number 12. For each month, the animals are shown in a cumulative fashion, i.e. even though animal 1-19 had to be killed at month 5 because of the size of the tumor, it stays on the chart.*

Last year we suggested that this was an aberrant result and suggested that it may have been due to the low incidence of tumors, quality problems with NMU or shipping stress to the animals. However, in the current year, we have repeated this experiment and obtained what looks to be the same result. This experiment has only reached month 6 so far, but the results seem clear:

Animal group	1	2	3	4
Animals with tumors	19/24	11/24	7/24	9/24

(same animal groups as above)

i.e. that the recently pregnant group are more susceptible. It does look, however, as if both U-PRL and S179D PRL are protective against this effect. This is a new and potentially important finding, but one that we will not be able to publish until we can explain why our control results differ from previous results in the literature. This will have to be done under the auspices of a

new grant. We suspect that the timing of carcinogen administration is the key and that the mammary gland is particularly susceptible to carcinogen administration during involution. The animals treated with U-PRL would not have been going through involution.

Statement of work item 2 is therefore as complete as we can make it during this funding period and has produced evidence consistent with a protective effect of S179D PRL, albeit in a way not previously predicted. Support from this grant will be acknowledged when the data can be published.

#### **Statement of work item (3).**

We have been successful in establishing a rat mammary gland organ culture system. Other investigators have used mouse mammary glands for this purpose and the mouse system is well established (24). No one, however, has reported success with a rat system. We have persevered and used the rat system because it is a better animal model for the study of human breast cancer and a better animal model for endocrine-related cancer. The challenges for the rat organ system include the larger size of the gland and the difficulty in ensuring access of administered polypeptide hormone to the ductal tree as it sits on a larger fat pad. Having conducted age and dose-response studies in this system, we have concluded that the use of glands from 21-day-old virgin females and incubation in 15  $\mu$ g/ml of each PRL will give us dependable results. We use the contralateral gland as the control for each animal. The number 4 glands have been used for whole mount analysis, and the number 5 glands for regular histology.

Using this system we have investigated direct effects of U-PRL and S179D PRL on the glands *in vitro*. Glands are incubated for 5 days in insulin (I, 5  $\mu$ g/ml), aldosterone (A, 1  $\mu$ g/ml), progesterone (P, 1  $\mu$ g/ml) and estrogen (E, 1 ng/ml) with and without added U-PRL or S179D PRL. In the absence of either PRL, ductal growth and branching occurs (Figure 1 appended separately). In the presence of U-PRL, branching appears normal and alveoli develop (Figure 2). In the presence of S179D PRL, branching is inhibited and the ducts are smaller (Figure 3). Because the major role of P in development of the mammary gland is the stimulation of branching within the ductal tree (25), it appears that S179D PRL, not only inhibits the effects of U-PRL, but also the effects of P. This is a very important new finding demonstrating in this second system that PRL directly affects ductal morphogenesis and that not all of PRL's effects on ducts are mediated through circulating P. It is very interesting that S179D PRL can oppose the effect of P on branching, but the mechanism by which this occurs is unknown at present. We have begun to further investigate this and other steroid-PRL interactions using mammary cell lines in culture. What we have found so far is that S179D PRL upregulates expression of the vitamin D receptor in both HC11 cells and MCF7 cells (see below and appended manuscript for more details), has no significant effect on the expression of E receptors in MCF7 cells (HC11 cells do not express the E receptor) and ongoing studies are examining the effect on P receptor. These issues will be explored further when additional grant funds are obtained.

Statement of work item 3 has been completed and has shown that S179D PRL has a direct inhibitory effect on ductal growth.

#### **Statement of work item (4)**

This *in vivo* experiment was designed to test whether exposure of virgin animals to S179D PRL would reduce tumor incidence in response to NMU. Rats were implanted with 28 day Alzet minipumps delivering 24  $\mu$ g/24h 2 weeks prior to NMU administration. In other words, the

animals were pretreated with PRL and treatment continued for 2 weeks after exposure to carcinogen. This experiment is in month 5 and has produced very exciting results:

Animal group	1	2	3
Animals with tumors	11/15	14/15**	5/15

1 = control animals receiving saline

2 = animals which received U-PRL

3 = animals which received S179D PRL

\*\* two animals sacrificed due to tumor burden

The tumors in group 3 also appeared later and are currently smaller. The experiment has 4 more months to run, at which time final data analysis will include total number of animals bearing tumors, time to tumor appearance, size of tumors and histopathology. At this time, however, it is clear that S179D PRL does afford some protection against breast cancer development. It is hoped that this will turn out to be permanent and not just a delay in tumor appearance once the final numbers are obtained.

Statement of work item 4 is therefore currently ongoing. Repetition of the pregnancy experiment put us behind schedule on this experiment and caused us to have to look for additional funds to complete the project. The completion is being funded by cancer research gift funds to the PI.

#### **Statement of work item (5)**

This item has several parts. The first is histopathological analysis of some of the tumors from item 4. Clearly, this has yet to be done. The second part was endocrine analysis from these animals. This we did during the first month when the animals were being treated with the PRLs. We found no significant differences among the three groups in the levels of E, P or corticosterone. We did, however, find that administration of U-PRL to the rats elevated their own production of PRL, whereas S179D PRL was without effect. This effect therefore can contribute to the tumor-promoting effect of U-PRL, but is not relevant to tumor refractoriness induced by S179D PRL.

#### **Derivative experiments and results**

##### **Part 1**

Presentation of the data produced under the auspices of this grant has made us aware that many investigators in the field have difficulty with the concept that two different PRLs can use the same PRL receptor(s) and yet have different effects on a cell. In other words, that U-PRL can promote cell growth, while S179D PRL not only inhibits the action of U-PRL on growth, but is also capable of inducing tissue-specific gene expression. To overcome this problem, we have investigated the signaling pathways activated after treatment with U-PRL or S179D PRL. This part of the project, being a derivative part of the study, was partially funded from other sources. These experiments were conducted using a normal rodent mammary cell line and are published in *Biochemistry* (see appended published manuscript # 3). HC11 cells are unusual among mammary cells *in vitro* because they retain their ability to respond to PRL by inducing  $\beta$ -casein gene expression. They therefore represent a model system in which we can investigate the differential signaling of U-PRL and S-PRL. The current consensus is that the main signaling

pathways for PRL are the Jak 2-Stat 5 pathway and the MAP kinase pathway (reviewed in 26). There are, however, many more signaling molecules that are activated to some degree in a variety of tissues (reviewed in 26). From our experimentation, we were able to demonstrate that U-PRL primarily activated the Jak 2-Stat 5 pathway and that S179D PRL primarily activated the MAP kinase pathway, although both PRLs used both pathways to some extent. Figure 2 in the attached manuscript illustrates very similar Jak 2 activation with U-PRL or S179D PRL while Figure 3 illustrates very different Stat 5a tyrosine phosphorylation as a result. Even with reduced tyrosine phosphorylation in response to S179D PRL, however, electromobility shift assays showed that very similar complexes were produced between nuclear proteins and an oligonucleotide equivalent to the Stat 5 binding site of  $\beta$ -casein and that most of the complexes contained Stat 5a and not Stat 5b (Figure 4). Phosphoaminoacid analysis of Stat 5 showed a higher phosphoserine content in response to S179D PRL (Figure 5). Serine phosphorylation of other Stats has been shown to increase transcriptional activity (27) and hence this may also be the case for Stat 5a and the  $\beta$ -casein gene. Analysis of MAP kinase signaling demonstrated that S179D PRL was the better activator of ERKs 1 and 2 and that a 7-day incubation in S179D PRL upregulated ERK signaling almost 3 fold (Figure 7), while a 7-day incubation in U-PRL upregulated Stat 5a activation 2 fold. When an inhibitor of MAP kinase, PD 98059, was included in the incubations, it inhibited the amount of  $\beta$ -casein expression seen in the S179D PRL-treated cells that was over and above that seen with U-PRL, but had no effect on U-PRL-stimulated  $\beta$ -casein gene expression or the equivalent portion of the S179D PRL response (Figure 8). Incubation in S179D PRL for 7 days also markedly upregulated expression of the short PRL receptor (Figure 9). We propose that U-PRL interacts with the long and short form of the PRL receptor such that signaling occurs primarily through Jak 2 and Stat 5 with a small amount of MAP kinase activation produced at both the long and short receptor. The different conformation of the receptors brought about by the binding of S179D PRL, however, results in reduced Stat 5a signaling and increased MAP kinase signaling. As a consequence of upregulation of the short receptor in response to S179D PRL, the MAP kinase signaling is increased. Signaling through MAP kinase results in superior  $\beta$ -casein gene expression. Thus, we have demonstrated that U-PRL and S179D PRL primarily use different signaling pathways (although both are required) and that long-term incubation in one PRL or the other can exaggerate the difference. This helps us and others understand how one molecule can inhibit U-PRL-mediated effects while stimulating other effects in the same cells.

## Part 2

In collaboration with another DAMD grantee, Linda Schuler at the University of Wisconsin, we have also investigated the ability of S179D PRL to inhibit the growth of the human breast cancer cell line, MCF7. This is primarily her study and so will only be described in very general terms. Further detail can be obtained from the attached submitted manuscript #2. Work in the Schuler lab demonstrated that S179D PRL inhibited growth of MCF 7 cells. In MCF7 cells, it competed very effectively with U-PRL to block U-PRL-induced proliferation. It also blocked Stat 5 tyrosine phosphorylation and the accumulation of cyclin D1 in response to U-PRL. In MCF7 cells in which the endogenous PRL was knocked out, 10 ng/ml S179D PRL reduced responses to 100 ng/ml U-PRL by 50%. Thus S179D PRL has proved to be a most efficacious growth antagonist for breast cancer cells. This finding has additional significance since it represents independent validation of growth antagonism results from this lab and was

done using preparations of S179D PRL from this lab and from the Goffin laboratory in France. This manuscript is currently submitted.

#### Part 3

We have also been working with both breast cancer cell lines and with the HC11 cells to further our understanding of the interactions between steroid hormones and S179D PRL (as mentioned above) and to further our understanding of the mechanism of action of S179D PRL. The most complete story at present shows that S179D PRL upregulates expression of the vitamin D receptor (figures 5 and 6 in attached submitted manuscript # 1) and the cell cycle-inhibiting protein, p21 (figures 1 and 2), whereas U-PRL upregulates cyclin D1 (figure 3) and the kinase it regulates to promote the cell cycle, cyclin-dependent kinase 4 (figure 4). This occurs in both MCF 7 cells and HC11 cells. S179D PRL both inhibits cell cycle progression and blocks proliferative signaling from U-PRL and may make the cells more sensitive to growth inhibition by vitamin D. The results using HC11 cells have been submitted for publication. Ongoing studies are further investigating the vitamin D sensitivity issue which may result in a most effective joint therapy with S179D PRL and vitamin D. In addition, we have found that S179D PRL blocks the ability of E to stimulate the growth of MCF 7 cells. When these cells were grown in medium containing 5% charcoal/dextran stripped horse serum, they double their number in response to 10 nM E during a 5 day period. S179D PRL (1 $\mu$ g/ml) given concurrently or for 24 hours prior to E treatment, blocked this response (figure 4 of non-manuscript-associated figures). This, however, does not involve downregulation of the E receptor as assessed by Western blot (data not shown). Thus S179D PRL has direct effects on cell cycle regulating proteins and also inhibits the ability of U-PRL (which stimulates growth through other cell cycle-regulating proteins) and E and perhaps P (the latter based on the ductal branching study) to stimulate growth. Further investigation of this result must await further funding.

#### Part 4

We have also collaborated with another DAMD grantee, Chris Ormandy from the Garvan institute in Sydney. Studies in his lab have duplicated our original in vivo studies which showed S179D PRL to inhibit the growth of the mammary gland during pregnancy. He has gone on to further investigate the effects of S179D PRL by using microarray analysis. Briefly since once again these are primarily his results, all data are consistent with the blockade of proliferation by S179D PRL in the mammary gland. Part of this work was reported by Chris Ormandy at the Era of Hope meeting in September 2002 and will shortly be submitted for publication.

#### Part 5

Other derivative studies include analysis of the kinase that normally phosphorylates PRL. Because of the specific effects of phosphorylated PRL and its potential therapeutic impact on breast cancer, it is also important to understand the regulation of phosphorylation in the pituitary. Such understanding may create opportunities for therapy which will elevate endogenous production of the beneficial form of the hormone. This study was conducted in collaboration with Jolinda Traugh of this institution who is an expert on p21-activated protein kinases (PAKs). The studies were afforded by using pituitaries from control rats in other experiments. From these pituitaries, we produced prolactin secretory granule preparations which were then analyzed for their PAK activity and their ability to phosphorylate endogenous and added PRL. Figure 3 in the attached published manuscript # 2 shows the presence of gamma PAK in the secretory granules

and figure 2 demonstrates that this activity phosphorylates both endogenous and added PRL at serine 179.

### KEY RESEARCH ACCOMPLISHMENTS

- Established that U-PRL stimulates ductal and alveolar growth in the pregnant and non-pregnant mammary gland and that at least part of the effect on ducts is direct and not mediated through circulating P.
- Established that S179D PRL inhibits ductal branching and overall growth in the pregnant and non-pregnant mammary gland and that this is a direct effect on the ducts.
- Established that S179D PRL not only inhibited growth, but also promoted a measure of differentiation in the mammary gland.
- Established that U-PRL and S179D PRL initiate their different effects on the mammary gland by differential use of the two major signaling pathways. S179D PRL not only inhibits signaling from U-PRL, but generates an alternate signal. This explains how S179D PRL can be effective at lower than 1:1 ratios with U-PRL. Although S179D PRL upregulates expression of the short PRL receptor and this increases signaling via the MAPkinase pathway, there is at present no other reason to suggest that the two PRLs primarily use different receptors.
- Established that S179D PRL is protective against NMU-induced tumors in virgin animals.
- Produced evidence suggesting that involution of the mammary gland makes it more susceptible to carcinogen assault.
- Through collaboration, have reproduced both our *in vivo* and *in vitro* results demonstrating that S179D PRL is a growth antagonist in the mammary gland.
- Established that S179D PRL inhibits cell cycle progression both by inhibiting signaling from U-PRL and by upregulating the cell cycle inhibiting protein, p21.
- Established that S179D PRL also upregulates expression of the vitamin D receptor, a result which suggests that S179D may make the cells more sensitive to the growth inhibiting effects of vitamin D.
- Have produced some evidence that S179D PRL blocks the effects of both E and P on mammary epithelial cells.
- Have established that gamma p21-activated protein kinase is the kinase in secretory granules that normally phosphorylates PRL.

### REPORTABLE OUTCOMES

#### Presentations at Learned Societies

- Symposium presentation at the 2001 Endocrine Society meeting, Denver, CO, entitled "Signaling and Biological Activity of a Molecular Mimic of Phosphorylated Prolactin". Abstract S4-2. p 28.
- Poster presentation of "Differential modulation of the expression of the long and short form of the PRL receptor suggests that the short form of the receptor does not act as a dominant negative for signaling resulting in  $\beta$ -casein expression". 2001 Endocrine Society meeting, Denver, CO. Abstract P2-219. p 340.

- Oral presentation by Wei Wu at the 2002 Endocrine Society meeting, San Francisco, CA, entitled "Differential Signaling of Unmodified PRL and S179D PRL in HC11 Cells" Abstract OR15-1.p 84.
- Co-authorship on a presentation from Chris Ormandy's group entitled "Galanin regulation of mammary lobuloalveolar development" given at the Era of Hope meeting, Orlando, Fl, 2002. Abstract P2-20.
- Oral presentation at the Era of Hope meeting entitled "Pseudophosphorylated Prolactin (S179D PRL) inhibits growth and promotes differentiation in the rat mammary gland". Abstract P2-27.
- Poster Presentation of "Pseudophosphorylated prolactin inhibits mammary cell growth and upregulates p21(waf1) and vitamin D receptor via activation of ERK1/2" at the Endocrine Society meeting in Philadelphia, 2003. Abstract P3-118, p 503.

#### Published papers

- 1)Kuo CB, Wu W, Xu X, Yang L, Coss D, Birdsall B, Nasseri D and Walker AM. Pseudophosphorylated prolactin (S179D PRL) inhibits growth and promotes  $\beta$ -casein expression in the rat mammary gland. *Cell and Tissue Res.*309:429-437 (2002).
- 2) Tuazon PT, Lorenson MY, Walker AM and Traugh JA. P21-activated protein kinase gamma-PAK in pituitary secretory granules phosphorylates prolactin. *FEBS Lett* 515:84-88 (2002).
- 3)Wu W, Coss D, Lorenson MY, Kuo CB, Xu X and Walker AM. Different biological effects of unmodified prolactin and a molecular mimic of phosphorylated prolactin involve different signaling pathways. *Biochemistry* 42: 7561-7570 (2003).

#### Submitted manuscripts

- Wu W, Tan D, Chen Y-H, Lorenson MY and Walker AM. Pseudophosphorylated prolactin up-regulates p21 and vitamin D receptor.
- Schroeder MD, Brockman JL, Walker AM and Schuler LA. Inhibition of PRL-induced proliferative signals in breast cancer by a molecular mimic of phosphorylated PRL, S179D PRL.

#### Personnel supported by this grant

- Wei Wu, postdoc. Dr Wu, who was promoted to Assistant Research Biomedical Scientist during the period of this grant, has submitted two of his own grant applications based on his work conducted under the auspices of this grant.  
Three graduate students have participated in this project at different times, depending on the skill sets required and the timing of their graduation:
  - Yen-Hao Chen, graduate student researcher. Work on this grant will eventually constitute a part of Mr Chen's PhD dissertation.
  - Djurdjica Coss, graduate student researcher. Work on this grant contributed to Dr Coss' PhD dissertation. Dr Coss is now a postdoctoral fellow in Pamela Mellon's lab at the University of California, San Diego.
  - Xiaolei Xu, graduate student researcher. Work on this grant contributed to Dr Xu's PhD dissertation. Dr Xu is now a postdoctoral fellow at the City of Hope Cancer Research Center in Duarte, California.
- Mayza Vue was an undergraduate work-study lab assistant.

## CONCLUSIONS

Work on this grant has substantiated our hypothesis about the differential roles of U-PRL and S179D PRL in the mammary gland. In other words, that U-PRL promotes growth of ducts and alveoli, while S179D PRL inhibits ductal growth and promotes differentiated function. These differential effects are brought about by actions directly on the mammary gland. Since most breast cancers are ductal in origin, this further suggests that S179D PRL would inhibit development and/or progression of the disease. This suggestion is borne out by the two *in vivo* experiments which show S179D PRL to afford some protection against the development of NMU-induced tumors.

Although we previously believed that S179D PRL only inhibited the effect of U-PRL on ductal growth, further work has illustrated that S179D PRL can also inhibit the effect of progesterone on mammary ductal branching and the effect of estrogen on cell proliferation. Branch points in ducts are areas of cell proliferation and matrix destruction. Both cell proliferation and matrix destruction are important for tumor growth and the metastatic potential of tumors.

Analysis of the differential signaling initiated by the two forms of PRL showed that the form of PRL, which stimulates growth, mostly uses the Jak 2-Stat 5 pathway while, contrary to expectations, the form of PRL that stimulates differentiation mostly uses the MAP kinase pathway. Long term incubations (days) in the different PRLs upregulated signaling *via* their respective pathways. This suggests that long term changes in the ratio of U-PRL to P-PRL around the duct would be of immense importance in the balance between proliferation and differentiation. Signaling pathways are also potential points of intervention in the fight against tumor formation and spread.

Analysis of effects on cell cycle have shown that U-PRL, signaling through Jak 2-Stat 5, upregulates cyclin D1 and the kinase it regulates to promote the cell cycle and that S179D PRL inhibits this signaling and also upregulates p21, signaling through MAP kinase, to inhibit progression though the cell cycle.

An agent which inhibits proliferation, potentially inhibits matrix destruction and promotes differentiation in the mammary gland, has the potential to be an important therapeutic for the prevention and/or treatment of breast cancer.

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**APPENDIX CONTENTS**

**FIGURES 1-4**

**FIGURE LEGENDS**

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**SUBMITTED MANUSCRIPTS -2**

### FIGURE LEGENDS

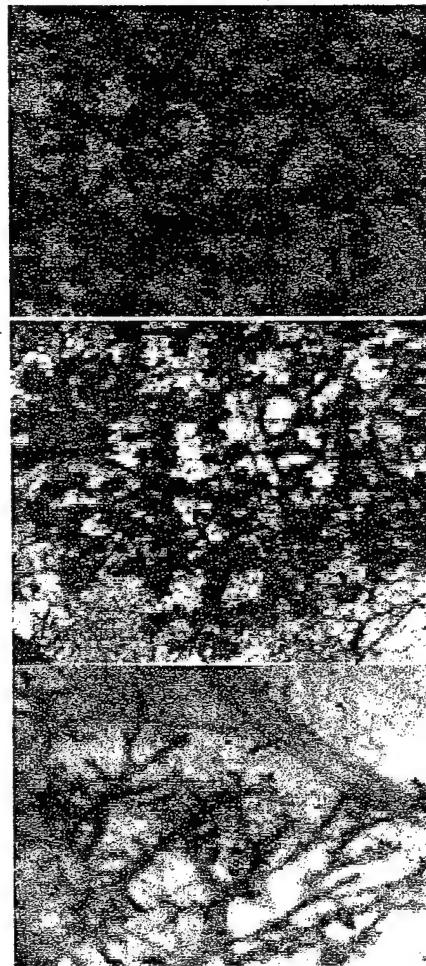
These legends are for the non-manuscript-associated figures presented first in the appendix. Figures 1-3 were submitted as color illustrations last year.

Figure 1: Control mammary explant cultured for 7 days in I, A, P and E. Note branching and therefore the network appearance of the ductal tree.

Figure 2: Mammary explant additionally exposed to U-PRL. Note the development of alveoli which is superimposed on a normal network of ducts.

Figure 3: Mammary gland explant additionally exposed to S179D PRL. Note the reduced branching and smaller ducts.

Figure 4: S179D PRL blocks estrogen stimulation of MCF7 cell proliferation. MCF7 cells, plated at 4000 cells per well, were incubated in two solutions during the experiment. The first incubation was for 48 hours and the second for 72 hours. All incubations were in DMEM with 5% charcoal/dextran-stripped horse serum. C = control to which the estrogen diluent was added; S = S179D PRL at 1  $\mu$ g/ml; E2 = estrogen at 10 nM; M = medium alone; OD = optical density using the MTS assay, which is a measure of cell number. The MTS assay was conducted in medium without any serum. Note the ability of S179D PRL to block the effect of E2. From this and other similar experiments, we know that S179D PRL is also effective when given concurrently with E2 on days 1-2. Of special interest is the duration of S179D's effect. In the third bar, one can see that a 2-day incubation in S179D PRL essentially completely blocked a proliferative response to E2 in the subsequent 3 days. Data are presented with SEM.

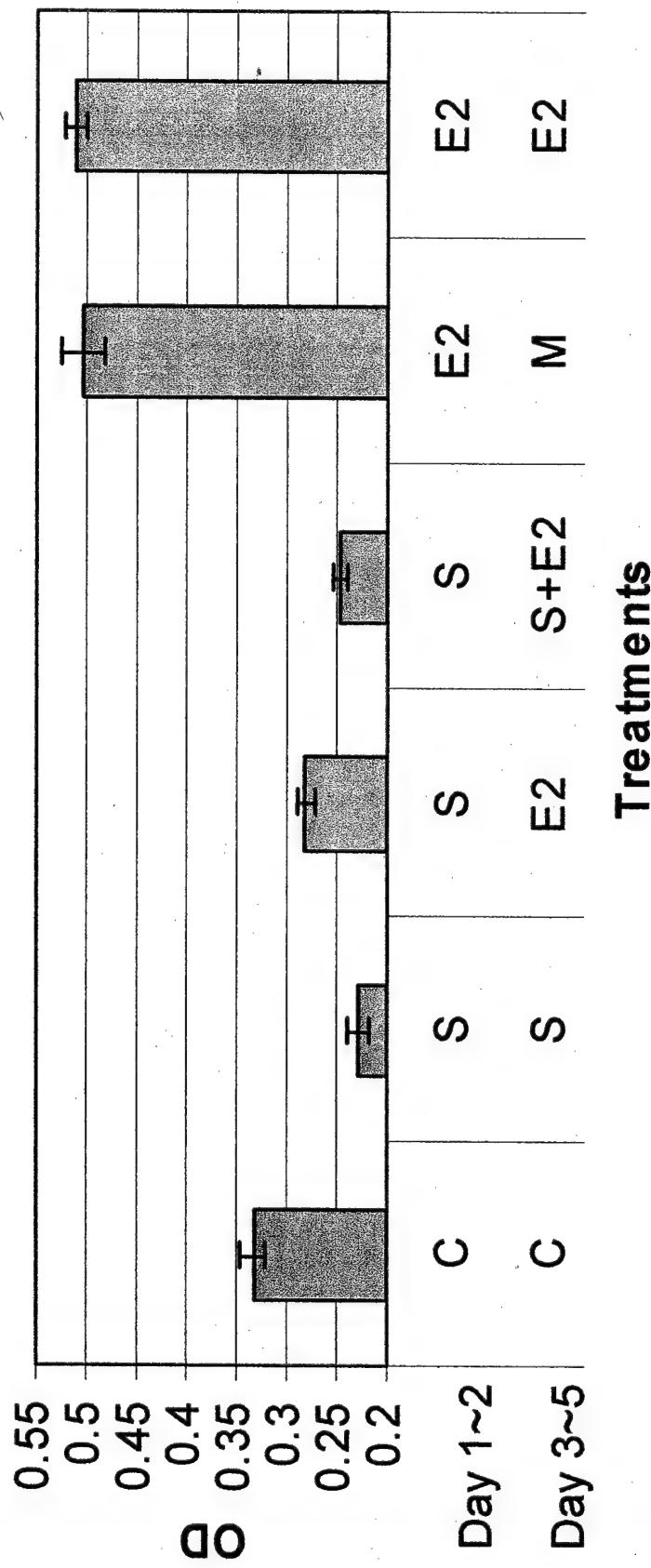


**Figure 1**

**Figure 2**

**Figure 3**

**Figure 4**  
**MCF-7 Cell Proliferation Assay**



**Abstract Preview**  
**SIGNALLING AND BIOLOGICAL ACTIVITY OF A MOLECULAR MIMIC OF PHOSPHORYLATED PROLACTIN.**

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PRL has been described as important for both growth and differentiation in the mammary gland, but its precise contribution to each of these processes has proved difficult to ascertain. In part, this is due to complications introduced into experimental protocols by the luteotropic action of PRL in rodent models and in part, in our opinion, because PRL has been thought of as a single substance. PRL has been shown to be posttranslationally modified in several ways. In rodents, a single posttranslational modification predominates. Thus unmodified PRL (U-PRL) and phosphorylated PRL (P-PRL) together make up 98-100% of the PRL released from the pituitary. In order to study the specific roles of U- and P-PRL, we have produced recombinant versions of each. Recombinant P-PRL was produced by molecular mimicry--substituting an aspartate for the normally phosphorylated serine, thereby producing S179D PRL. Physiological analyses have shown that the ratio of U- to P-PRL changes with time during pregnancy and lactation. Because both PRL forms are always present, we have studied their roles *in vivo* by adjusting the relative ratio of each form in the animal. Administration of either PRL to pregnant and non-pregnant animals was without effect on the levels of progesterone (P), estrogen and corticosterone. Additional U-PRL during pregnancy stimulated ductal and alveolar growth. Additional S179D PRL inhibited ductal and alveolar growth while promoting the formation of alveoli and  $\beta$ -casein gene expression. These effects required the formation of alveoli and  $\beta$ -casein gene expression. These effects required neither the levels of P normal to pregnancy nor placental products since they were duplicated in non-pregnant animals. Elevated P, however, increased the growth-promoting effects of U-PRL in the ducts. Thus it appears that the effects of U-PRL tend towards growth in the mammary gland while those of P-PRL tend towards differentiation. To examine this in more detail, we have assessed the individual roles of the two PRLs on signal transduction in HC11 cells. Compared with U-PRL, S179D PRL resulted in decreased tyrosine, and increased serine, phosphorylation of STAT 5a, increased  $\beta$ -casein gene expression and increased expression of the short PRL receptor suggesting that differentiative effects of P-PRL may be mediated in part through interaction with the short form of the PRL receptor.

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**Abstract Preview**

**DIFFERENTIAL MODULATION OF THE EXPRESSION OF THE LONG AND SHORT FORM OF THE PRL RECEPTOR AND -CASEIN BY UNMODIFIED PRL AND A MOLECULAR MIMIC OF PHOSPHORYLATED PRL SUGGESTS THAT THE SHORT FORM OF THE RECEPTOR DOES NOT ACT AS A DOMINANT NEGATIVE FOR SIGNALLING RESULTING IN -CASEIN EXPRESSION.**

W. Wu<sup>1</sup>, X. Xu<sup>1</sup> and A.M. Walker<sup>1</sup>. <sup>1</sup>Biomedical Sciences, University of California, Riverside, CA, United States

HCII mouse mammary cells were grown in RPMI 1640 containing 10% FBS, 5  $\mu$ g/ml insulin and 10 ng/ml epidermal growth factor (EGF). Once confluent, the medium was changed daily. On the third day post-confluence, the growth medium was removed, the cells washed 5 times to remove EGF and then incubated in RPMI 1640 supplemented with 10% charcoal-stripped horse serum, 10  $\mu$ g/ml insulin and 1  $\mu$ g/ml hydrocortisone for 24h (priming medium). At the end of the 24h period, the priming medium was changed and then further supplemented with 0 or 1  $\mu$ g/ml unmodified recombinant human PRL (WT PRL) or the molecular mimic of human phosphorylated PRL (S179D PRL). Exposure to the PRLs continued for 7 days, during which time the medium was changed daily. Total RNA was extracted, treated with DNase I, run on a 1% agarose formaldehyde gel at 10  $\mu$ g/lane and then transferred to a nylon filter. Probes for Northerns were produced from purified RT-PCR products of rat PRL (399 bp) and mouse  $\beta$ -casein (201 bp) mRNA. The probe for the PRL receptor recognized domains common to all forms. All image data were normalized for loading and transfer using a 1.2 kb cDNA of mouse 18S rRNA. Treatment with No PRL, WT PRL or S179D PRL resulted in different ratios of short (S) to long (L) receptor and different degrees of  $\beta$ -casein gene expression. Thus an S:L ratio of 5.2 in the cells not exposed to PRL resulted in a  $\beta$ -casein expression level designated as 1. Treatment with WT PRL elevated the S:L (by decreasing L) to 6.04 and  $\beta$ -casein expression to 10.8. Treatment with S179D PRL elevated the S:L (by decreasing L and elevating S) to 6.96 and the  $\beta$ -casein to 19.23. Thus a linear relationship was obtained whereby  $\beta$ -casein expression was a direct function of S:L. This result argues against the idea that S functions as a dominant negative to  $\beta$ -casein expression and in fact supports the idea that an increase in S:L expression is directly responsible for  $\beta$ -casein upregulation. USARMC #BC 990711

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**BASIC - Growth Hormone & Prolactin Signal Transduction****OR15-1****Differential Signaling of Unmodified PRL and S179D PRL in HC11 Cells.**

Wei Wu\*, Djurdjica Coss<sup>1</sup>, Benson Kuo<sup>1</sup>, Xiaolei Xu<sup>1</sup>, Ameae M Walker<sup>1</sup>. <sup>1</sup>Div of Biomed Scis, Univ of California, Riverside, CA.

The anterior pituitary releases both unmodified PRL (U-PRL) and phosphorylated PRL (P-PRL). Previous work has shown that P-PRL antagonizes the growth-promoting activities of U-PRL and that this effect is duplicated by a molecular mimic of P-PRL, S179D PRL. At the same time, S179D PRL is a super PRL agonist with regard to  $\beta$ -casein gene expression. In this study, we have examined the differential signaling of recombinant U-PRL and S179D PRL in HC11 cells. HC11 cells were grown to confluence in RPMI/10% FBS/5  $\mu$ g/ml insulin/10 ng/ml EGF, maintained at confluence by daily media changes for 2-3 days, primed for 24 h by removal of EGF plus exchange of FBS for charcoal-stripped HS and the addition of 1  $\mu$ g/ml hydrocortisone and then exposed in fresh priming medium to the PRLs.

A 15 min incubation at 5  $\mu$ g/ml led to substantial activation of Jak 2 and Stat 5a by U-PRL and an essentially equivalent Jak 2 activation by S179D PRL. The latter, however, was accompanied by a much reduced activation of Stat 5a. EMSA analysis using the  $\beta$ -casein GAS site showed both PRLs to cause equivalent binding of nuclear proteins and that most of what bound was Stat 5a. Phosphoamino acid analysis of Stat 5a showed S179D PRL to double the amount of serine phosphorylation versus that seen with U-PRL. Analysis of the MAPK pathway showed U-PRL capable of activation of ERKs 1 & 2, but that signaling via ERKs 1 & 2 was very much greater with S179D PRL. A 7 day incubation in both PRLs at 1  $\mu$ g/ml increased  $\beta$ -casein gene expression as judged by Northern blot, but S179D PRL caused a 5 fold increase over that seen with U-PRL. The increase over that seen with U-PRL was blocked by PD 98059. After 7 days of treatment with S179D PRL, expression of the short PRL receptor was doubled and signaling showed a greater dependence on the MAPK pathway (2.9 fold increase in ERK 1 & 2 phosphorylation), whereas treatment with U-PRL produced very little change. We conclude that U-PRL signals primarily through Jak 2/Stat 5 whereas S179D PRL signals primarily through the MAPK pathway although both PRLs utilize both pathways to some extent. In addition, it appears that activation of the MAPK pathway leads to greater serine phosphorylation of Stat 5 and increased  $\beta$ -casein gene expression. Coincident increases in the expression of the short PRL receptor and utilization of the MAPK pathway suggest that S179D PRL increases MAPK signaling via an upregulation of the short PRL receptor. DAMD 17-00-0810

## Abstract 4

### GALANIN REGULATION OF MAMMARY LOBULOALVEOLAR DEVELOPMENT

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Galanin is a trophic neuropeptide involved in neuronal development and neuroendocrine regulation, and is also expressed by breast cancer cells indicating a possible role in mammopoiesis. Galanin and galanin receptors were found to be differentially expressed in the mammary gland during pregnancy. Null mutation of the galanin gene resulted in reduced lobuloalveolar development and lactational failure accompanied by decreased pituitary secretion of prolactin and an increase in relative phosphorylated prolactin levels. Treatment of wild-type mice with a molecular mimic of phosphoprolactin inhibited alveolar growth and caused lactational failure. Unmodified prolactin restored lactation in galanin knockout mice, however, rescue of lobuloalveolar development was incomplete. Transplantation of galanin knockout mammary epithelia to wild-type hosts demonstrated galanin does not have an essential autocrine/ paracrine role in mammary development. A direct endocrine role was demonstrated by culture of whole mammary gland explants, where addition of galanin resulted in an increase in size and a four-fold increase in the number of lobuloalveoli produced by lactogenic hormones alone. These data demonstrate that galanin is critical for mammary lobuloalveolar development and differentiation during pregnancy, acting directly on the mammary gland and also indirectly as a positive regulator of Prl secretion and phosphorylation during mammopoiesis.

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The U.S. Army Medical Research and Materiel Command under DAMD17-99-1-9185 supported this work.

## Abstract 5

### PSEUDOPHOSPHORYLATED PROLACTIN (S179D PRL) INHIBITS GROWTH AND PROMOTES DIFFERENTIATION IN THE RAT MAMMARY GLAND

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Prolactin (PRL) has long been recognized as a hormone having both proliferative and differentiative activities in the mammary gland. Current theory proposes that it is the co-existing steroid environment which dictates whether PRL is mainly proliferative, as it is during pregnancy, or mainly differentiative, as it is during lactation. Our hypothesis, on the other hand, suggests that the form of PRL is also important in that it dictates a primarily proliferative versus primarily differentiative response, with unmodified PRL (U-PRL) producing the former and phosphoPRL, the latter. To test this hypothesis, recombinant versions of each PRL were administered to rat dams throughout pregnancy at a rate resulting in circulating concentrations of 50 ng/ml. Measurement of progesterone, estradiol and corticosterone showed no effect of the administered PRLs on these other mammatropic hormones. Histological and morphometric analysis showed U-PRL to cause an increase in gland size ( $102 \pm 7 \text{ mm}^3$ , U-PRL v  $67 \pm 5 \text{ mm}^3$ , control,  $p < 0.01$ ), while the molecular mimic of phosphoPRL, S179D PRL, decreased gland size ( $40.2 \pm 4.6 \text{ mm}^3$ ,  $p < 0.05$ ). The number of pup implantation sites was indistinguishable among groups ( $13 \pm 1$ ), as was the level of expression of placental lactogen II mRNA in placentae from day 19.5 of gestation (Northern blot). In addition to an overall decrease in size of the S179D PRL-treated mammary gland, there was a decrease in area occupied by alveoli to almost half the control level ( $p < 0.001$ ). This resulted in lactational failure. In contrast, Northern analysis of  $\beta$ -casein expression normalized to 18s RNA demonstrated decreased expression in mammary glands from the U-PRL-treated dams and increased expression in the S179D PRL-treated dams ( $p < 0.05$ ). *In vitro* studies with the rodent mammary cell line, HC11, confirmed the superior ability of S179D PRL to promote  $\beta$ -casein gene expression (7 fold more potent than U-PRL,  $p < 0.01$ ). Further experiments with non-pregnant animals confirmed that effects on the mammary gland were not secondary to changes in placental lactogens and that pregnancy levels of progesterone were not required. We conclude that U-PRL is primarily proliferative and that S179D PRL is primarily differentiative in the pregnant mammary gland, although there is some degree of cross activation. Ongoing experiments include 1) analysis of the interplay among these two PRLs and the other mammatropic hormones using mammary tissue explants, and 2) testing of the hypothesis that exposure to additional phosphorylated PRL during pregnancy would enhance pregnancy-related refractoriness to carcinogenesis.

## Abstract 6

P3-118

### Pseudophosphorylated Prolactin Inhibits Mammary Cell Growth and Upregulates p21(waf1) and Vitamin D Receptor Via Activation of ERK1/2.

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We have previously established that a molecular mimic of phosphorylated prolactin (S179D PRL) inhibits pregnancy-induced growth of rat mammary glands, independently of effects on other circulating mammogenic hormones (1). We reasoned that this inhibition of growth by S179D PRL may be due to the induction of cell cycle regulatory proteins. In this regard, others have reported that upregulation and activation of p21 via vitamin D resulted in cell cycle arrest, differentiation and apoptosis in human breast cancer MCF7 cells (2,3). We therefore investigated the effects of S179D PRL on mammary cell growth and the expression of p21 and the vitamin D receptor (VDR). When mouse HC11 and human MCF7 cells were incubated for 3 days in RPMI 1640 and 5% charcoal-stripped horse serum (medium changed daily), results showed that S179D PRL reduced both HC11 and MCF7 cell number in a dose dependent fashion, with 50% inhibition of cell number occurring at 100 ng/ml in both cell lines. A similar incubation at 1 $\mu$ g/ml S179D PRL increased protein expression of p21 by 40 and 60% ( $p<0.05$ ), and VDR by 50 and 100% ( $p<0.01$ ) in HC11 and MCF7 cells, respectively. The increased amount of VDR was all located in the nucleus, as one would expect if the VDR was responsible for the upregulation of p21. Since we have previously shown that S179D PRL activates ERK 1/2 (4) and numerous studies from other laboratories have shown that sustained activation of ERK1/2 leads to upregulation of p21 in some cell types (5), we investigated the potential role of MAP kinases in signaling from S179D PRL to p21 and VDR upregulation. The MAP kinase inhibitor, PD98059 at 25  $\mu$ M completely blocked upregulation of p21 and VDR expression, thereby implicating the MAP kinase signaling pathway in this response.

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2. Breast Cancer Res Treat 2001;67:157-168.
3. Endocr relat Cancer 2002;9:45-59.
4. Endocrine Soc Abstracts 2002; p84.
5. Anticancer Res 2001; 21:499-504.

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REGULAR ARTICLE

C. Benson Kuo · Wei Wu · Xiaolei Xu · Lili Yang  
Cyndi Chen · Djurdjica Coss · Ben Birdsall  
Dorsa Nasser · Ameae M. Walker

## Pseudophosphorylated prolactin (S179D PRL) inhibits growth and promotes $\beta$ -casein gene expression in the rat mammary gland

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**Abstract** We have investigated the individual roles of unmodified prolactin (U-PRL) and a mimic of phosphorylated PRL (S179D PRL) in mammary development. Recombinant versions of the PRLs were delivered to rats throughout pregnancy at a rate of 6  $\mu$ g/24 h per rat and to non-pregnant females at a rate of 24  $\mu$ g/24 h per rat. Measurement of progesterone, corticosterone, and estradiol showed no effect of the administered PRLs on the levels of these other mammatropic hormones. Histological and morphometric analysis showed U-PRL to cause mammary growth, whereas S179D PRL inhibited growth. Molecular analysis demonstrated decreased  $\beta$ -casein expression in the mammary glands of the U-PRL-treated animals at term and increased  $\beta$ -casein expression in the mammary glands of the S179D PRL-treated animals. Superior  $\beta$ -casein gene expression in response to S179D PRL versus U-PRL was confirmed in HC11 cells. We conclude that U-PRL is important for growth, whereas S179D PRL promotes at least one measure of differentiated function in the mammary gland.

**Keywords** Unmodified prolactin · Phosphorylated prolactin · Duct · Alveolus ·  $\beta$  casein · Rat (Sprague Dawley)

### Introduction

Prolactin (PRL) has long been described as a hormone important for both growth and differentiation in the mammary gland, but its precise contribution to each of

these processes has been difficult to ascertain. This is attributable, in part, to complications introduced into experimental protocols by the luteotropic action of PRL in rodent models (Hsueh et al. 1984) and, in part, in our opinion, because PRL has been thought of as a single substance. In this study, we have used an experimental approach that maintains the normal progesterone levels of pregnancy to test the effects of increased unmodified PRL and phosphorylated PRL on mammary gland development. PRL is produced in a variety of post-translationally modified forms. We have focused our attention on the individual biological roles of unmodified PRL and phosphorylated PRL, because these two forms between them constitute 98%–100% of secreted pituitary PRL in the rodent (Oetting and Walker 1986; Ho et al. 1993a, 1993b), which serves as our experimental model. They also have been demonstrated to have distinct biological activities (Ho et al. 1989; Krown et al. 1992; Wang and Walker 1993; Coss et al. 1999, 2000; Yang et al. 2001), the proportion of each released from the pituitary is physiologically regulated (Ho et al. 1993a, 1993b), and phosphorylated PRL has been found in all species thus far examined (reviewed by Lorenson and Walker 2001). Standard preparations of PRL, such as those distributed by the NIDDK, contain a mixture of unmodified and phosphorylated PRL (Krown et al. 1992; Wang and Walker 1993). Any biological activity observed as a result of treatment with these preparations therefore represents an aggregate activity related to the relative proportions of the unmodified and phosphorylated PRL present (Krown et al. 1992; Wang and Walker 1993).

In order to determine the individual activities of unmodified and phosphorylated PRL in the mammary gland, we have administered recombinant versions of each to pregnant and non-pregnant animals, thereby altering the normal ratio of the different PRL forms in the animal. In the case of phosphorylated PRL, we have produced a molecular mimic by substituting an aspartate residue for the normally phosphorylated serine, thereby producing S179D PRL (Wang et al. 1996; Chen et al. 1998). Aspartate mimicry of serine phosphorylation is

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used extensively in studies of enzymes activated or deactivated by phosphorylation, and in several instances, extensive structural analyses have confirmed complete three-dimensional and functional mimicry (Thorsness and Koshland 1987; Wittekind et al. 1989). The recombinant wild-type hormone (U-PRL) is identical to unmodified PRL with the exception, like S179D PRL, of an N-terminal extra methionine (Chen et al. 1998). The S179D PRL effectively mimics the naturally phosphorylated molecule by acting, like phosphorylated PRL, as an extremely effective antagonist to U-PRL-induced Nb2 cell proliferation (Wang and Walker 1993; Chen et al. 1998).

While S179D PRL is an antagonist to growth at physiological ratios with U-PRL and at concentrations physiologically relevant for a given tissue (Chen et al. 1998; Yang et al. 2001), it is a partial agonist when used alone at concentrations 100-fold those of U-PRL (Bernichttein et al. 2001). This finding has lead to some controversy about the molecule. There are however a number of examples of partial agonists that act physiologically as antagonists in some tissues and as very effective agonists in others. Tamoxifen and Raloxifen, for example, act as antagonists in the breast and as agonists in the bone (MacGregor and Jordan 1998). In a similar manner, S179D PRL acts as an antagonist in the lung and thymus (Chen et al. 1998; Yang et al. 2001), but like U-PRL in the bone (Coss et al. 2000). Indeed, it seems unlikely that the body would produce an absolute antagonist. Thus, it is not entirely unexpected that the biology of S179D PRL, as a mimic of phosphorylated PRL, is more complicated than simple antagonism of the actions of U-PRL. The most important reason for using the molecular mimic rather than the naturally phosphorylated molecule is in order to prevent the possible interconversion of phosphorylated PRL to U-PRL by body phosphatases. Even though this is a very slow process (Krown et al. 1992; Wang and Walker 1993), conversion would severely complicate interpretation of results. The aim of the present study has been to determine the individual roles of the two forms of PRL in the mammary gland by analyzing effects on structural development and expression of the major milk protein,  $\beta$ -casein.

## Materials and methods

### Animal experiments

Virgin female Sprague-Dawley rats ( $n=35$ , 16 weeks old) were divided into four groups. Five rats served as non-pregnant controls, 10 rats as normal pregnant controls, 10 rats as recipients of U-PRL, and 10 rats as recipients of S179D PRL. Alzet minipumps (Alza, Palo Alto, Calif.) delivering 6  $\mu$ g PRL/24 h per rat for 28 days were implanted subcutaneously the morning after vaginal plug observation. This was considered as being day 0.5 of pregnancy. On day 6.5, blood was obtained from the tails and collected into heparinized tubes. At term, dams were separated from their pups for 20 h prior to sacrifice to standardize the histological appearance of the glands in each group. After sacrifice, the inguinal mammary glands were dissected out, measured, and processed for whole-mount or histological examination. Size was calculated by

multiplying the length by the average width by the average depth. In an essentially duplicate experiment, blood samples ( $\sim 1$  ml) were taken from the tails on days 6.5 and 11.5 and trunk blood from the neck after decapitation at day 19.5. No animal was bled more than once from the tail in order to keep stresses during pregnancy to a minimum. At the time of death (day 19.5 of pregnancy, five dams in each group; or day 21.5 shortly after pup delivery, five dams in each group), inguinal mammary glands were snap-frozen in liquid N<sub>2</sub> for later RNA extraction. In a third and fourth experiment, non-pregnant females were treated with 24  $\mu$ g PRLs/24 h per rat for 4 days. By using a higher dose for a shorter interval, it was thought likely that individual responses to each form of PRL would be exaggerated and therefore become clearer. At the time of sacrifice, trunk blood was collected, and the inguinal mammary glands were again processed either for whole-mount or regular histology. All animal procedures were approved by the University of California, Riverside Campus Committee on Laboratory Animal Care and were in accord with NIH guidelines.

### Recombinant PRLs

Both recombinant human U-PRL and S179D PRL were produced and characterized as previously described (Chen et al. 1998). Both proteins were expressed and purified in parallel and were expressed at similar levels (Chen et al. 1998). The preparations were then tested for their activity in an Nb2 bioassay. U-PRL promoted Nb2 cell proliferation, whereas S179D PRL (like naturally phosphorylated PRL; Wang and Walker 1993) antagonized this (Chen et al. 1998). The PRL preparations were concentrated to 1 mg/ml saline by using Amicon Centriprep (Amicon, Danvers, Mass.) and loaded into model 2004 (first two experiments) or 2001 (third and fourth experiments) Alzet minipumps.

### Histological analysis

Mammary glands were fixed in periodate-lysine-parafomaldehyde fixative (McLean and Nakane 1974) at 4°C overnight. The fixed tissue was dehydrated in a graded ethanol series, cleared in Hemo De, and then embedded in Paraplast. Sections (6  $\mu$ m thick) were cut and stained with hematoxylin and eosin. For morphometric analysis of the glands from non-pregnant animals, entire mammary glands were serially sectioned. Stained sections were viewed at a constant magnification by using a PAXIT digital image system (Midwest Information Systems, Franklin Park, Ill.), and the glands were measured on a screen. Every duct and associated dense stroma was measured on each section. The area occupied by alveoli was also measured. For morphometric analysis of term pregnant glands, three mid-gland sections from five animals in each group were analyzed. In each case, a random view at the same low magnification was photographed, and the area on each photograph occupied by alveoli versus stroma plus ducts, was determined. For whole-mounts, glands were spread on glass and then fixed in Carnoy's solution (60% ethanol, 30% chloroform, 10% glacial acetic acid) for 60 min at room temperature. They were then washed in ethanol and defatted overnight in acetone. Defatted glands were then rehydrated and stained with carmine alum overnight at 4°C, dehydrated, and cleared in methyl salicylate before mounting.

### Hormone assays

All steroid hormones were measured by radioimmune assay with a kit from Diagnostic Products (Diagnostic Products Coat-a-Count, Los Angeles, Calif.). Progesterone levels were measured in serum (trunk blood) or plasma (tail blood). Previous work had demonstrated equivalent recognition of progesterone in rat serum and plasma with heparin as the anticoagulant (Coss et al. 2000). All results presented in a single table were produced in the same

assay. Errors were therefore limited to intra-assay variation. The coefficient of intra-assay variation for this assay was 6.7% in our hands. Only trunk blood samples were assayed for corticosterone. As for progesterone, all results in the relevant table were produced in the same assay. The coefficient of intra-assay variation was 6.3% in our hands. Total estradiol levels were measured in trunk blood. The coefficient of intra-assay variation was 5.7% in our hands.

#### Northern blot analysis for $\beta$ -casein gene expression

Total RNA was isolated from mammary tissue or HC11 cells by using the Trizol RNA reagent (Gibco BRL, N.Y.). The isolated RNA was treated with DNase I (Gibco BRL, Gaithersburg, Md.). Equal amounts of RNA (10  $\mu$ g) from control and test samples were loaded on a 1.0% agarose formaldehyde gel. The gels were run at 60 V for 3–5 h. RNA was blotted onto nylon filters (Micron Separations, Westboro, Mass.) by capillary transfer with 10 $\times$ SSC (1 $\times$ SSC=150 mM NaCl, 15 mM sodium citrate, pH 7.0) and fixed by ultraviolet cross-linking. The 201-bp probe used for hybridization was from a mouse  $\beta$ -casein cDNA polymerase chain reaction product. The primers were: 5'-CCC GTC CCA CAA AAC ATC C-3' (forward); 5'-ATT AGC AAG ACT GGC AAG GCT G-3' (reverse). The probe was labeled with 50  $\mu$ Ci [ $\alpha$ - $^{32}$ P] dCTP (ICN Biomedicals, Costa Mesa, Calif.) by using a DECA Prime II DNA Labeling Kit (Ambion, Austin, Tex.). The labeled probes were separated by ProbeQuant G-50 Micro Columns (Amersham Pharmacia Biotech, Piscataway, N.J.). After a 2-h prehybridization at 65°C with the hybridization solution (25 mM Na<sub>2</sub>HPO<sub>4</sub>, pH 7.2, 1 mM EDTA, 7% SDS), hybridizations were carried out at 65°C for 16–24 h. The filters were then washed in alternating solutions of 20 mM Na<sub>2</sub>HPO<sub>4</sub>, pH 7.2, 1 mM EDTA, 5% SDS and then 20 mM Na<sub>2</sub>HPO<sub>4</sub>, pH 7.2, 1 mM EDTA, 1% SDS for a total of three times in each. Filters were exposed to Fuji medical X-ray film (Fuji Medical Systems, Stamford, Conn.) for 1–7 days at -70°C. Probe-stripping was performed by heating the nylon filter at 95°C for 10–30 min in a solution of 10 mM TRIS-HCl, pH 8.0, 1 mM EDTA, 1% SDS. A mouse 18S rRNA cDNA fragment (DECA template-18S-mouse, 1212 bp; Ambion) was used to normalize for errors in RNA loading and transfer. A Kodak 1D Image Analysis System was used for quantification (Eastman Kodak, Rochester, N.Y.).

#### Effect of U-PRL and S179D PRL on $\beta$ -casein gene expression in HC11 cells

RPMI 1640 basal medium (Gibco BRL, Grand Island, N.Y.) containing 2 mM L-glutamine and 2 g/l NaHCO<sub>3</sub> served as a basal medium. HC11 cells, a cloned mouse mammary epithelial cell line (Ball et al. 1988), were grown in RPMI 1640 growth medium containing 10% fetal calf serum (Gibco BRL, Grand Island, N.Y.), 5  $\mu$ g/ml insulin (Sigma, St. Louis, Mo.), 10 ng/ml epidermal growth factor (Gibco BRL, Gaithersburg, Md.), 100 U/ml penicillin, and 100  $\mu$ g/ml streptomycin. Once HC11 cells became confluent, they were grown for three more days in growth medium. The medium was changed daily. On the third day post-confluence, the growth medium was removed, and the cells washed five times with RPMI 1640 basal medium. The cells were refed with priming medium. Priming medium was RPMI 1640 basal medium supplemented with 10% charcoal-stripped horse serum (Cocalico Biologicals, Reamstown, Pa.), 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin, 10  $\mu$ g/ml insulin, and 1  $\mu$ g/ml hydrocortisone (Sigma). The cells were kept in priming medium for 24 h. The cells were then refed with induction medium. The induction medium was priming medium to which 1  $\mu$ g/ml of the appropriate PRL was added. This protocol essentially follows Taverna et al. (1991). In the present study, cells were maintained in induction medium for 7 days and refed daily. The cells were collected for RNA isolation.

#### Statistical analysis

Analysis of variance was performed using the INSTAT program (GraphPAD Software, San Diego, Calif.). Post-tests comparing each potential pair of groups were performed. Bonferroni corrections were used to allow for more than one comparison against a single control group. A *P*-value less than 0.05 after Bonferroni correction was considered significant.

## Results

#### Pregnant animals

##### Gross observations

Treatment with U-PRL at 6  $\mu$ g/24 h per rat throughout pregnancy resulted in glands that were 1.5-fold the size of those from the untreated pregnant animals (102 $\pm$ 7 mm<sup>3</sup>, U-PRL; 67 $\pm$ 5 mm<sup>3</sup>, control; *P*<0.01). Treatment with S179D PRL at 6  $\mu$ g/24 h per rat throughout pregnancy, by contrast, resulted in glands that were 40% smaller (40.2 $\pm$ 4.6 mm<sup>3</sup>; *P*<0.05). As reported previously (Yang et al. 2001), the average number of pup implantation sites per animal (13 $\pm$ 1) was indistinguishable among groups.

##### Histological observations

Figure 1 shows equivalent sections of mammary glands from each group of animals from the first experiments. Treatment with U-PRL resulted in larger lobuloalveoli than those in the control pregnant animals, whereas treatment with S179D PRL resulted in smaller lobuloalveoli than in the controls. The area occupied by alveoli versus stroma and ducts was significantly different from controls in both treatment groups (Table 1). Of particular note was the finding that, in the 40% smaller glands of the S179D PRL-treated animals, the area occupied by lobules was reduced to almost half (*P*<0.001). In other words, a greater amount of intervening connective tissue was evident. This had resulted in lactational failure in previous experiments where this was monitored. A difference in morphological appearance of the milk/colostrum was also evident in the different groups. Thus, U-PRL treatment increased the number of lipid droplets, whereas S179D PRL treatment decreased the lipid content (compare D–F in Fig. 1).

#### Hormone levels

Table 2 shows progesterone levels to be unaltered by U-PRL or S179D PRL treatment throughout pregnancy, and estradiol and corticosterone levels to be unaltered on day 19.5 of pregnancy. *P*-values were always greater than 0.05.

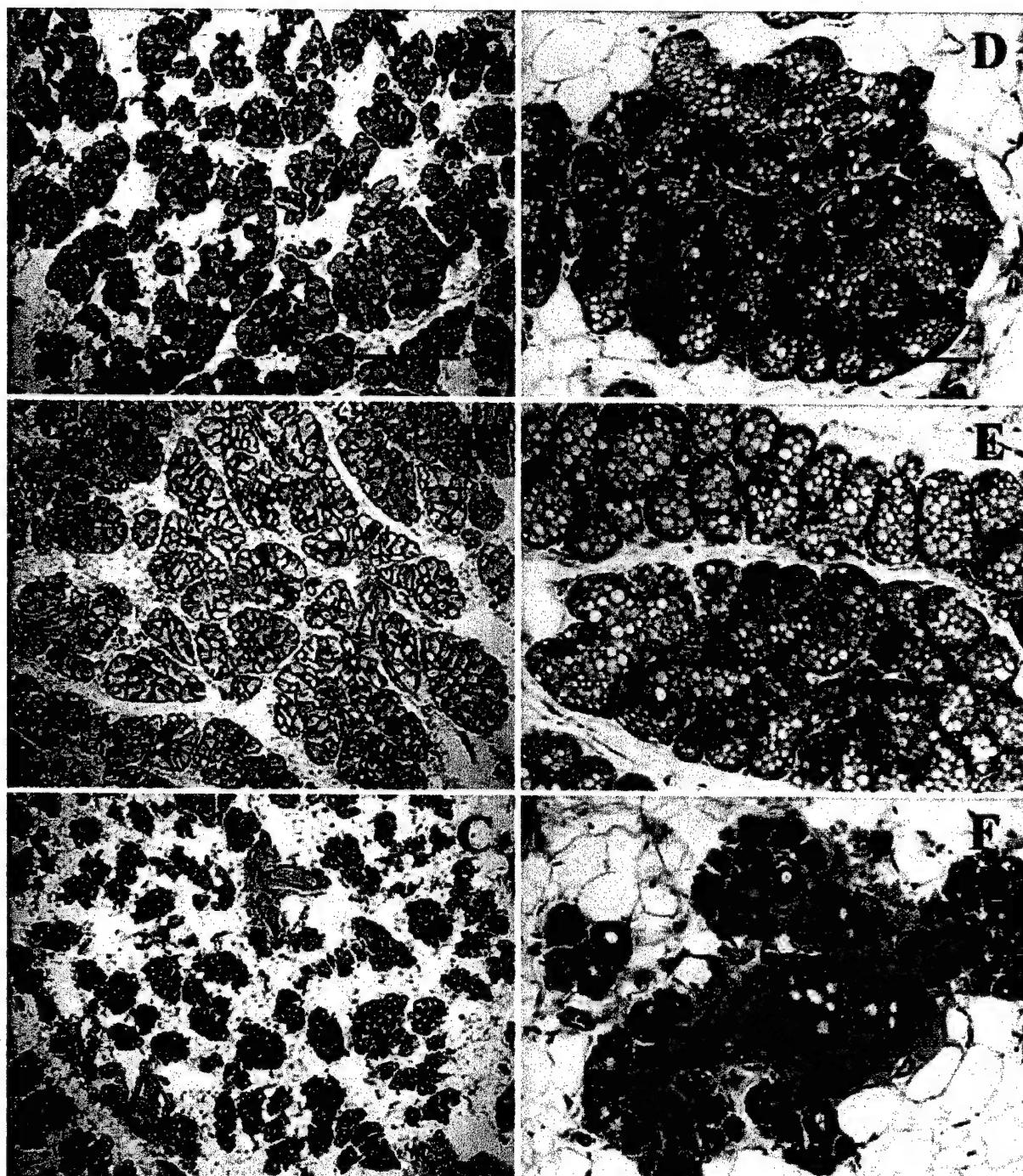


Fig. 1A-F Term mammary glands from each treatment group. A, D Control rats. B, E Rats treated with U-PRL. C, F Rats treated with S179D PRL. Note the large number of lipid droplets in E and

their relatively low abundance in F. Also note the proximity of the small alveoli to a relatively large duct in F. A-C  $\times 40$ , D-F  $\times 200$ . Bar 1 mm (A), 200  $\mu$ m (D)

#### $\beta$ -Casein expression

The result of Northern analysis for  $\beta$ -casein expression in the day 19.5 and 21.5 samples is shown in Fig. 2. Both time points gave the same result, and so the data were combined allowing for sufficient samples for ade-

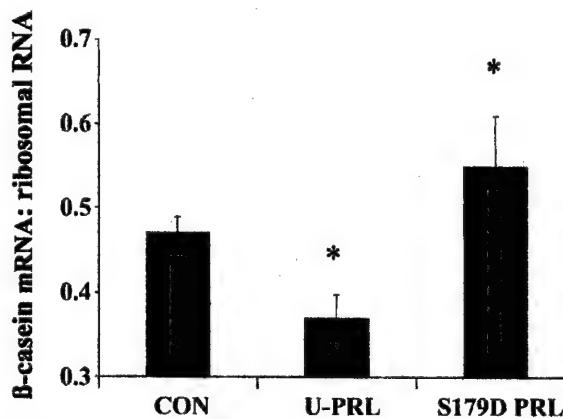
quate statistical analysis. U-PRL treatment caused reduced  $\beta$ -casein gene expression, whereas S179D PRL treatment caused an enhancement. Because treatment with the PRLs was over and above the animal's own lactogens, and because normalization to ribosomal RNA can be questioned in glands with different epithelial to

**Table 1** Area of gland section occupied by alveoli or stroma plus ducts on day 21.5 and expressed as a percentage of the total area. Data are expressed as the mean $\pm$ SE with  $n=15$  per group (significant difference: \* $P<0.05$ , \*\* $P<0.001$ )

Tissue	Day	Control	U-PRL	S179D PRL
Alveoli	21.5	67.5 $\pm$ 1.1*, **	72.3 $\pm$ 1.3*	38.3 $\pm$ 0.9**
Stroma plus ducts	21.5	32.5 $\pm$ 0.6	28 $\pm$ 0.9	61.7 $\pm$ 1.1

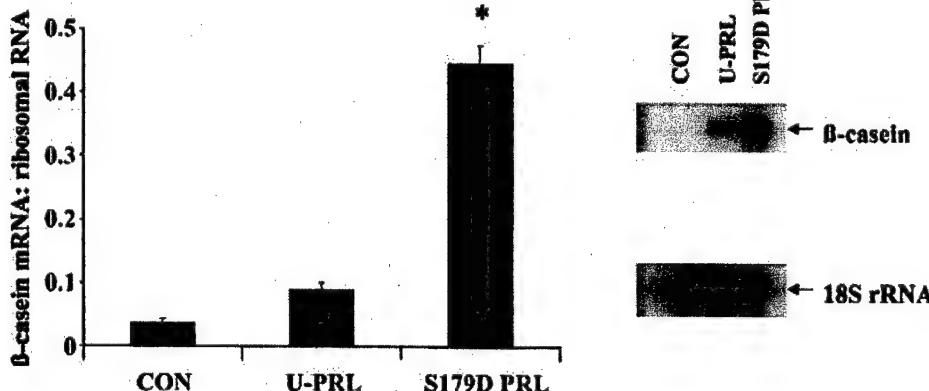
**Table 2** Levels of mammotrophic steroids during pregnancy in the three treatment groups of the second experiment. Data are expressed as the mean $\pm$ SE with  $n=5$  animals per time point and group. There were no statistically significant changes

Treatment	Day	Control	U-PRL	S179D PRL
Progesterone (ng/ml)	6.5	374 $\pm$ 37	351 $\pm$ 20	323 $\pm$ 28
	11.5	369 $\pm$ 20	380 $\pm$ 20	389 $\pm$ 43
	19.5	377 $\pm$ 23	283 $\pm$ 28	328 $\pm$ 18
Estradiol (pg/ml)	19.5	15 $\pm$ 1	14.3 $\pm$ 1	15.1 $\pm$ 2
Corticosterone (ng/ml)	19.5	138 $\pm$ 29	205 $\pm$ 62	169 $\pm$ 22



**Fig. 2** Northern analysis of  $\beta$ -casein mRNA expression in mammary glands at days 19.5 and at term ( $n=8$  rats per group). Amounts of  $\beta$ -casein mRNA are normalized to the amount of ribosomal RNA. Results are expressed as the mean $\pm$ SE (CON control rats receiving no additional PRL). Differences among groups were analyzed by ANOVA and individual *t*-tests with Bonferroni corrections. \* $P<0.05$  (significantly different from the control group)

**Fig. 3** Northern analysis of  $\beta$ -casein mRNA expression in HC11 cells in response to the various PRLs (CON control rats receiving no additional PRL). Amounts of  $\beta$ -casein mRNA are normalized to the amount of 18S RNA. Differences among groups were analyzed by ANOVA and individual *t*-tests with Bonferroni corrections. \* $P<0.001$  (significantly different versus the control and U-PRL groups; mean $\pm$ SE of five separate experiments). Inset: Representative blot



connective tissue ratios, we also examined the effects of the two PRLs on  $\beta$ -casein gene expression in the HC11 mammary cell line. Figure 3 clearly shows that S179D PRL is many-fold more effective at inducing  $\beta$ -casein gene expression than is U-PRL in the 7-day treatment period.

#### Non-pregnant animals

By performing similar experiments in non-pregnant animals, it was possible to test whether the effects observed on the pregnant mammary gland were secondary to effects on placental lactogens. In addition, we could ask whether pregnancy levels of progesterone were required to observe these effects. The histology of mammary glands from animals treated with the PRLs at 24  $\mu$ g/24 h per rat for 4 days is presented in Fig. 4. Both PRLs caused mammary development by comparison with the controls. However, U-PRL did this by promoting ductal and alveolar growth. S179D PRL, by contrast, produced smaller alveoli arising from smaller ducts. In other words, when S179D PRL was present in excess, it was a poor agonist for mammary growth but did result in some alveolar development. The overall picture is best illustrated by the whole-mount images shown in Fig. 5. Since these are of rat glands rather than of the much smaller mouse glands, whole-mount images have fewer elements in focus in a given image. Nevertheless, it is clear that the alveoli are smaller in the S179D PRL-treated glands. Because of the degree of development of these glands, it was not possible to illustrate the effects on ductal branching by using low-magnification whole-mount photographs. Instead, overall growth of the ductal tree was assessed by measuring ductal diameter. As the tree enlarges, so does the average diameter of the branches. Morphometric analysis of serial sections showed the ducts of the U-PRL treated glands to be 1.4-fold the diameter of the ducts in the S179D PRL treated and control glands (Table 3). The width of the associated dense stroma was however the same in both groups. Thus, effects on ductal growth do not seem to be secondary to proliferation of the associated dense stroma.

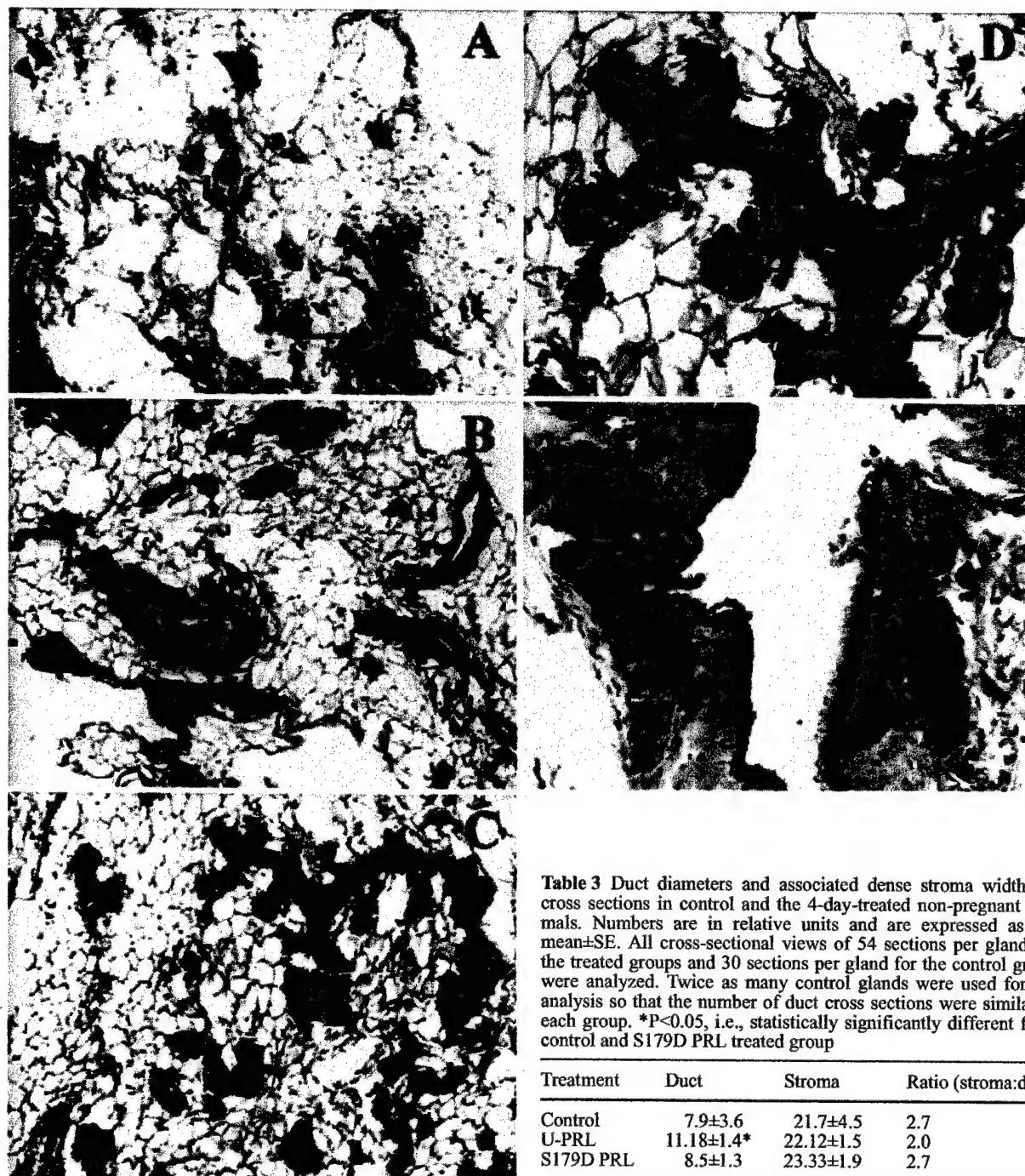


Fig. 4A-E Non-pregnant mammary glands treated with the various PRLs for 4 days. A Control rats. B Rats treated with U-PRL. C Rats treated with S179D PRL. D, E Higher magnification views of mammary glands from rats treated with S179D PRL. Note the many small alveoli closely associated with a relatively large duct in C-E. A-C  $\times 80$ , D, E  $\times 200$ . Bar 500  $\mu$ m (A), 200  $\mu$ m (D)

Table 4 shows no statistically significant effect of the two PRLs on corticosterone, progesterone, or estradiol levels in these non-pregnant animals. Trends were, if anything, toward equally reduced corticosterone and

Table 3 Duct diameters and associated dense stroma widths in cross sections in control and the 4-day-treated non-pregnant animals. Numbers are in relative units and are expressed as the mean  $\pm$  SE. All cross-sectional views of 54 sections per gland for the treated groups and 30 sections per gland for the control group were analyzed. Twice as many control glands were used for the analysis so that the number of duct cross sections were similar in each group. \* $P < 0.05$ , i.e., statistically significantly different from control and S179D PRL treated group

Treatment	Duct	Stroma	Ratio (stroma:duct)
Control	7.9 $\pm$ 3.6	21.7 $\pm$ 4.5	2.7
U-PRL	11.18 $\pm$ 1.4*	22.12 $\pm$ 1.5	2.0
S179D PRL	8.5 $\pm$ 1.3	23.33 $\pm$ 1.9	2.7

Table 4 Levels of progesterone, corticosterone, and estradiol in the 4- to 5-day-treated non-pregnant animals. Data are expressed as the mean  $\pm$  SE. These levels are not significantly different among groups ( $P > 0.05$ )

Treatment	Control	U-PRL	S179D PRL
Progesterone (ng/ml)	25.2 $\pm$ 8.0	14.6 $\pm$ 5.3	17.4 $\pm$ 4.4
Corticosterone (ng/ml)	331 $\pm$ 85	280 $\pm$ 32	256 $\pm$ 66
Estradiol (pg/ml)	17.2 $\pm$ 2.1	16 $\pm$ 1.9	18.3 $\pm$ 1.3



**Fig. 5A–C** Whole-mount glands at equivalent magnifications from non-pregnant animals not treated or treated for 5 days with the various PRLs. **A** Control. **B** Treatment with U-PRL. **C** Treatment with S179D PRL. Note the larger alveoli in **B** and the multiple smaller alveoli in **C**.  $\times 20$

progesterone with each PRL, i.e., toward an equal reduction in these other mammatropic hormones with both treatments.

## Discussion

Administration of additional U-PRL has markedly different effects on mammary gland histology from those seen following the administration of S179D PRL. We can conclude therefore that the effects are not attributable to a simple elevation in total PRL but are, indeed, specific to each form. Since there is no effect on progesterone, estradiol or corticosterone, we can conclude that the effects observed are not secondary to changes in these other mammatropic hormones. Similar effects in pregnant and non-pregnant animals demonstrate that these effects are not secondary to changes in placental lactogen. This was considered as a possibility because other investigators have demonstrated that mammary gland development is related to the number of developing pups (Nagasawa and Yanai 1971), and because the levels of placental lactogens are very much in excess of PRL in the later stages of pregnancy (Robertson and Friesen 1981). Placental lactogens are thought to function via the PRL receptor (Freemark et al. 1993). Similar effects in pregnant and non-pregnant animals also tell us that pregnancy levels of progesterone are not required in order to be able to see the differential activities of each form, although our current experiments do not address the question of the necessity for some progesterone or the promotion of the processes by progesterone. Progesterone has been shown to upregulate PRL receptors on rodent mammary epithelium (Edery et al. 1985) and hence is likely to make the system more responsive to PRL, in addition to having substantial and totally independent effects.

U-PRL significantly promotes ductal growth (as reflected in the diameter of ducts) in only 4 days at 24  $\mu\text{g}/24\text{ h}$  in non-pregnant animals. This rate of administration results in circulating levels of 200 ng/ml by day 4, although days 1–3 have lower amounts as the PRL from the mini pump slowly equilibrates with tissue and blood compartments (Coss et al. 2000). At only 6  $\mu\text{g}/24\text{ h}$ , or 50 ng/ml (Coss et al. 2000), administered U-PRL results in a 50% increase in the overall size of the mammary gland at term. Some of this size increase is attributable to the growth of lobuloalveoli, but some has to be the result of ductal growth in accord with the findings in non-pregnant animals. Since the gland as a whole is still contained within the fat pad, some general (as opposed to duct-associated dense) stromal proliferation is likely to have occurred. A similar concentration of circulating S179D PRL reduces the size of the mammary gland at term, i.e., it inhibits ductal growth and branching. In the short-term experiment, alveoli can be seen developing almost directly from large ducts in Fig. 4D, E, i.e., they appear to cap duct branch points and prevent their further development. At the same time, S179D PRL promotes  $\beta$ -casein gene expression. However, because the gland is too small, insufficient milk is produced to feed the pups.

When viewing the  $\beta$ -casein expression data from the pregnancy experiment, it is important to remember that the effect is caused by the administration of the recombinant PRLs over and above the rat's own PRL, which is a mixture of unmodified PRL and phosphorylated PRL. Changing the ratio by increasing U-PRL decreases  $\beta$ -casein gene expression, because it reduces the relative amount of phosphorylated PRL, which is a much better stimulator of  $\beta$ -casein expression. Thus, the pregnancy data are concordant with the effects of the individual PRLs on the HC11 cells.

From these results, it appears that U-PRL promotes overall growth of the mammary gland. S179D PRL, and presumably therefore phosphorylated PRL, acts as an antagonist to mammary growth but promotes an alveolar differentiated function. When used in high enough concentration, S179D PRL can also promote alveolar development. Whether this is partial agonism or promotion of differentiation remains to be established. Until now, the effects of PRL on growth versus differentiation in the mammary gland have been thought to be attributable to a change in the steroid environment between pregnancy and lactation. This certainly plays a major role, but it is also clear that the forms of PRL are important. In regard to the forms of PRL, we have previously shown an increase in the ratio of U-PRL to phosphorylated PRL to occur during the latter two-thirds of rodent pregnancy when the mammary gland is growing (Ho et al. 1993b). Just before parturition, there is a peak of PRL (Fliestra and Voogt 1997), which is high in phosphorylated PRL (unpublished data); phosphorylated PRL is very high in colostrum and milk (Ellis and Picciano 1993; Kacsoh et al. 1993), and the majority of PRL receptors are on the milk face of the mammary epithelium (Clevenger et al. 1995). Thus, the ontogeny of PRL forms during pregnancy and lactation is concordant with the observed effects of the individual PRL forms on the mammary gland; first, mostly growth, and then, an increase in  $\beta$ -casein gene expression.

Recent work utilizing a variety of mammary epithelial and stromal transplant recombinations from the PRL receptor knockout mouse and the progesterone receptor knockout mouse supports our findings of a role for a lactogen in both ductal and alveolar growth, although the ductal growth is deduced in these studies to be indirect via effects on progesterone (Ormandy et al. 2001). Whereas the transplant studies show that progesterone plays a very important role in ductal growth, transplant studies are qualitative and not quantitative and could easily have missed the additional, more minor, contribution of PRL itself. In this regard, a recent study by Sasaki et al. (2001) has also demonstrated co-operative effects of prolactin and progesterone during mouse mammary gland branching morphogenesis.

Our results showing the effects of PRL on ductal growth without effects on progesterone levels suggest a direct effect of PRL on the duct. Indeed, if there is any trend in the U-PRL-treated animals showing greater ductal growth, it is for a decrease in progesterone levels. A direct effect of PRL on ductal growth is very much in keeping with the presence of PRL receptors in ductal

epithelium (Ouhtit et al. 1993a). Alternatively or additionally, PRL may act indirectly via the stroma, although it is clear that the amount of dense stroma is not increased. Other investigators have implicated epidermal growth factor (Wiesen et al. 1999), transforming growth factor  $\beta$  (Daniel et al. 1996), hepatocyte growth factor (Soriano et al. 1998), insulin-like growth factor 1 (Kleiberg et al. 2000), and vascular endothelial growth factor (Pepper et al. 2000) as being stromal factors that positively influence ductal growth. Rodent stroma, however, has been reported to be devoid of PRL receptors that would be required to effect such an indirect stimulation (Meister et al. 1992; Ouhtit et al. 1993a, 1993b).

The different effects of the two forms of PRL in the mammary gland are probably the result of different signaling. These two forms of PRL have been shown to initiate different signaling cascades in Nb2 cells (Coss et al. 1999) and in HC11 cells (Wu et al. 2001). In HC11 cells, S179D PRL upregulates the short form of the PRL receptor and signals primarily through MAP kinase for superior  $\beta$ -casein gene expression (Wu et al. 2001). Some activation of STAT5 through the Jak2/STAT5 pathway, however, is essential.

In conclusion, we have clearly shown individual effects of the two forms of PRL on the mammary gland. U-PRL promotes growth whereas S179D PRL (pseudo-phosphorylated PRL) inhibits growth and promotes a differentiated function in this tissue. It is probably important that both forms are present throughout development of the mammary gland. Excesses of U-PRL might otherwise result in uncontrolled growth, whereas excesses of phosphorylated PRL would inhibit necessary growth and cell replacement. The proportion of U-PRL to phosphorylated PRL must change during development of the mammary gland in preparation for lactation, such that growth initially predominates and is later superceded by differentiated function. A substance such as S179D PRL, which inhibits growth and promotes a differentiated function, may have potential in the treatment or prevention of breast cancer.

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# p21-activated protein kinase $\gamma$ -PAK in pituitary secretory granules phosphorylates prolactin

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**Abstract** p21-activated protein kinase  $\gamma$ -PAK phosphorylates prolactin (PRL) in rat pituitary secretory granules on Ser-177 and on the equivalent site, Ser-179, in recombinant human PRL. This is shown by comparison of phosphopeptide maps with the human PRL mutant S179D.  $\gamma$ -PAK is present in rat and bovine granules as identified by in-gel phosphorylation of histone H4, and by immunoblotting. Thus, phosphorylation of PRL by  $\gamma$ -PAK in granules produces the PRL molecule that has been shown to antagonize the growth-promoting activity of unmodified PRL, and is consistent with the identified role of  $\gamma$ -PAK in the induction and maintenance of cytostasis. © 2002 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

**Key words:** Prolactin; Protein kinase; p21-activated protein kinase; Pituitary secretory granule; Phosphorylation; PAK

## 1. Introduction

$\gamma$ -PAK (Pak2) is a ubiquitous member of the family of p21-activated protein kinases (PAKs) which are activated by autophosphorylation following binding of the GTP-bound form of the small G-protein Cdc42 (for reviews see [1–5]).  $\gamma$ -PAK is expressed ubiquitously, in contrast to  $\alpha$ -PAK which is found mainly in brain, but is also found in other tissues, and  $\beta$ -PAK which is specifically expressed in brain [1–5].  $\gamma$ -PAK, but not  $\alpha$ -PAK or  $\beta$ -PAK, is activated by caspase cleavage during apoptosis [6,7]. Both  $\alpha$ - and  $\gamma$ -PAK are autophosphorylated and activated in vivo and in vitro directly by sphingosine [8,9].

$\alpha$ - and  $\gamma$ -PAK are highly homologous but appear to have different functions.  $\alpha$ -PAK is involved in growth, as it is activated by growth factors [10,11] and insulin [12], and is involved in the regulation of actin cytoskeletal reorganization [13–16].  $\gamma$ -PAK is activated in response to a variety of stimuli that induce stress or lead to cytostasis or cell death, including hyperosmolarity [17], DNA damaging drugs and UV and ionizing radiation [18].  $\gamma$ -PAK appears to be involved in maintaining cells in a non-dividing or cytostatic state [1,19].

$\gamma$ -PAK phosphorylates a variety of proteins such as histones

4 and 2B, translation initiation factors, myosin light chain from smooth muscle, myosin light chain kinase, myelin basic protein, avian and Rous sarcoma virus nucleocapsid protein, and Abelson tyrosine kinase (c-Abl) (for review see [1]). The variety of substrates suggests the involvement of  $\gamma$ -PAK in the regulation and possible coordination of cellular processes. Regulation of biological functions by phosphorylation with  $\gamma$ -PAK has been shown for c-Abl [20], smooth muscle myosin light chain [21] and myosin light chain kinase [22], and Rous sarcoma virus nucleocapsid protein [23,24]. Using synthetic peptides as substrates, the recognition/phosphorylation determinants have been identified as K/RRXS where X is preferably an acidic amino acid, such as glutamate or aspartate [25].

Prolactin (PRL) is a polypeptide hormone (23 kDa) involved in maintaining normal reproductive functions as well as regulation of growth (for reviews, see [26,27]). PRL exists as multiple charged isoforms and is stored at high concentrations (50 mM) in pituitary secretory granules as osmotically inert structures [28,29]. Phosphorylation of PRL affects its biological functions [30,31]; in two different PRL-responsive cell lines, phosphorylated PRL has been shown to antagonize the growth-promoting effects of unmodified PRL [30,32]. The major phosphorylation site in vivo in rat PRL is serine 177 [33]; this corresponds to serine 179 in human and bovine PRL [34]. A mutant of human prolactin (S179D), where serine 179 is mutated to aspartate to mimic phosphorylated serine, is a highly effective antagonist to unmodified PRL [35]. In addition, it has been shown that phosphorylated PRL autoregulates PRL secretion in normal pituitary cells [36], and the phosphorylation states of the hormone released from the pituitary change during the estrous cycle [37] and during pregnancy and pseudopregnancy [38].

Previous studies show that purified rat PRL is phosphorylated in vitro by  $\gamma$ -PAK (originally known as PAK I) [39]. The in vivo phosphorylation site in rat PRL, serine 177/179 in the sequence RRDSHK [33], is in a highly conserved region among all PRLs [34] and contains the phosphorylation recognition determinant for  $\gamma$ -PAK. Here, we report that  $\gamma$ -PAK is present in rat and bovine pituitary secretory granules and phosphorylates Ser-177/179. The data suggest that  $\gamma$ -PAK regulates the growth-promoting activity of PRL in vivo.

## 2. Materials and methods

### 2.1. Materials

Histone 4 and histone IIAS were from Roche Biochemicals; cypermethrin was from LC Laboratories. Antibodies which react specifically with the regulatory domain of  $\gamma$ -PAK (N-19; SC 1872) or the catalytic domain of the PAK isoforms (C-19; SC 1519), peroxidase-

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**Abbreviations:** PAK, p21-activated protein kinase; PRL, prolactin; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid; GST, glutathione S-transferase

coupled secondary antibodies and protein A/G agarose were from Santa Cruz Biotechnology. RR-1 anti-PAK antibody, which reacts preferentially with endogenously activated  $\gamma$ -PAK [18], was obtained as previously described [19]. Cellulose thin-layer chromatography sheets were from Selecto Scientific and silica gel thin-layer chromatography sheets were from EM Science.

Glutathione S-transferase (GST)- $\gamma$ -PAK obtained by expression of the cDNA in insect cells (TN5B-4) was purified by binding to glutathione-Sepharose 4B and released with glutathione or by cleavage with thrombin as described previously [40]. GST-Cdc42 [41] and wild-type human PRL and the S179D mutant [35] were expressed in *Escherichia coli* and purified as described.

#### 2.2. Isolation of prolactin secretory granules

Prolactin secretory granules from bovine and rat were obtained as previously described [42,43]. Briefly, the production of rat granules involved homogenization in 0.32 M sucrose, removal of debris and nuclei by low-speed centrifugation, pelleting of organelles by centrifugation for 30 min at 15000  $\times g$ , and puromycin treatment of the pellet which resulted in the detachment of ribosomes from rough microsomes and lysis of contaminating growth hormone granules. The puromycin-treated material was loaded onto discontinuous sucrose gradients to yield a prolactin secretory granule fraction relatively free of contaminants, as indicated by ultrastructural and biochemical criteria [43]. The secretory granules were washed in high salt to remove proteins adsorbed on the cytoplasmic face of the granules, then pelleted in 0.32 M sucrose containing 5 mM MgCl<sub>2</sub> and 1.0 M KCl; the pellet was resuspended in 0.5 ml of 0.32 M sucrose solution. As described earlier, two-dimensional polyacrylamide gel electrophoresis and electron microscopic analyses of the subcellular fraction served as controls for purity [43]. Analysis of 5' nucleotidase as a plasma membrane marker, and succinate cytochrome *c* reductase as a mitochondrial marker, demonstrated maximal contamination of 2.7% and 1.35%, respectively [44].

#### 2.3. Phosphorylation of prolactin

In order to solubilize the limiting granule membrane and the proteins and to monomerize the multimeric structure of PRL, secretory granules (5–10  $\mu$ g) were pre-incubated at 37°C for 50–60 min in 12  $\mu$ l of buffer A (50 mM Tris-HCl, pH 7.5, 75 mM 2-mercaptoethanol, 7.5 mM ethylenediaminetetraacetic acid (EDTA), 0.02% Triton X-100) containing protease inhibitors, aprotinin and leupeptin (0.17 mg/ml) and the phosphatase inhibitor cypermethrin (0.4  $\mu$ M). Phosphorylation of PRL (10–20  $\mu$ g of granule) was carried out in 30  $\mu$ l reaction mixtures containing 20 mM Tris-HCl, pH 7.5, 8 mM MgCl<sub>2</sub>, 3 mM EDTA, 30 mM 2-mercaptoethanol, 0.008% Triton X-100, 0.2 mM [ $\gamma$ -<sup>32</sup>P]ATP (2000 cpm/pmol), 0.16  $\mu$ M cypermethrin, 0.07 mg/ml of aprotinin and leupeptin, and GST- $\gamma$ -PAK (0.2  $\mu$ g) activated with Cdc42 (GTP $\gamma$ S) [40]. Incubation was for 30 min at 30°C. Reactions were terminated by addition of 10  $\mu$ l of 100 mM ATP and sodium dodecyl sulfate (SDS) sample buffer. Following SDS polyacrylamide gel electrophoresis (SDS-PAGE), phosphorylated proteins were detected by autoradiography on X-ray film or with a phosphorimager.

Purified recombinant wild-type PRL and the mutant S179D (1.0  $\mu$ g) were phosphorylated as described above, except that there was no pre-incubation and Triton X-100 was omitted from buffer A.

#### 2.4. Tryptic phosphopeptide mapping and phosphoamino acid analysis

Phosphorylated PRL was excised from SDS polyacrylamide gels and extensively digested with trypsin and analyzed by two-dimensional phosphopeptide mapping and phosphoamino acid analysis [45].

#### 2.5. Detection of $\gamma$ -PAK activity by in-gel phosphorylation of histone

Proteins in PRL granules from rat (10  $\mu$ g) and bovine (20  $\mu$ g) were separated on 10% polyacrylamide gels cast with 0.2 mg/ml of histone H1AS; following electrophoresis, the proteins in the gel were denatured and renatured and the protein kinase activity was assayed in-gel as described [19].

#### 2.6. Western blotting

Proteins in PRL granules from rat (10  $\mu$ g) and bovine (20  $\mu$ g) pituitary glands were resolved by SDS-PAGE on a 10% gel and were electrophoretically transferred to polyvinylidene fluoride membranes. The membranes were blocked using 5% milk in Tris-buffered saline containing 0.1% Tween 20 and probed with antibodies (N-19 or

RR-1) which react specifically with the regulatory domain of  $\gamma$ -PAK, or C-19 which reacts preferentially with the catalytic domain of  $\gamma$ -PAK as described previously [18,19]. Antibody binding was detected using peroxidase-conjugated secondary antibodies and visualized with ECL chemiluminescence reaction reagents.

### 3. Results

#### 3.1. $\gamma$ -PAK phosphorylates PRL on serine 177/179

To determine whether PRL in secretory granules was phosphorylated by  $\gamma$ -PAK, rat granules were pre-incubated in the presence of Triton X-100, EDTA and reducing agent; this opened the granule membrane, allowing access to [ $\gamma$ -<sup>32</sup>P]ATP, solubilizing the semicrystalline proteins and converting the multimeric structure of PRL into monomers. The solubilized proteins were analyzed as substrates for  $\gamma$ -PAK by incubation with [ $\gamma$ -<sup>32</sup>P]ATP and Mg<sup>2+</sup> in the presence or absence of active  $\gamma$ -PAK. A low level of phosphorylation of rat PRL was observed when the extract was incubated alone (Fig. 1A). Upon addition of exogenous  $\gamma$ -PAK activated by preincubation with Cdc42(GTP $\gamma$ S), phosphorylation of PRL was increased 15–20-fold over that observed with endogenous protein kinase(s).

Previously, Ser-179 was shown to be the phosphorylation site involved in conferring antagonistic properties to phosphorylated human PRL, as compared to non-phosphorylated PRL. To determine whether  $\gamma$ -PAK phosphorylated this site, purified wild-type human PRL and the mutant S179D were examined as substrates for  $\gamma$ -PAK; both were phosphorylated,

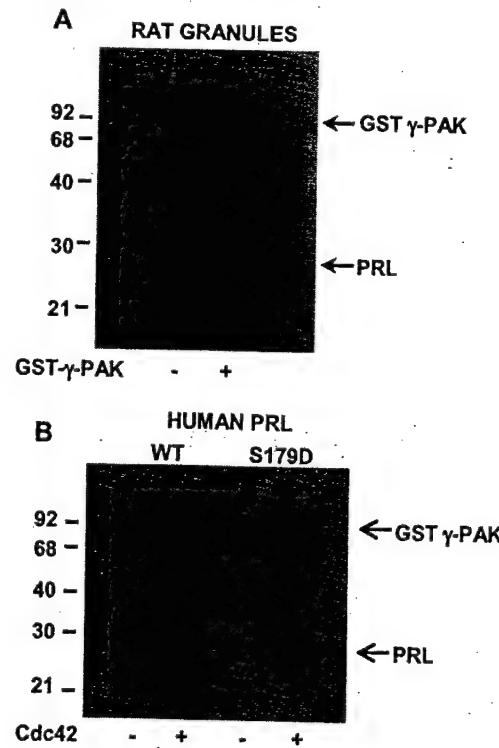


Fig. 1. Phosphorylation of PRL by  $\gamma$ -PAK. A: Solubilized protein from rat secretory granules (5  $\mu$ g) was incubated with [ $\gamma$ -<sup>32</sup>P]ATP in the presence or absence of activated GST- $\gamma$ -PAK as described in Section 2. B: Purified recombinant human PRL or the mutant S179D (1.0  $\mu$ g) phosphorylated with GST- $\gamma$ -PAK (0.2  $\mu$ g) in the presence or absence of Cdc42 (GTP $\gamma$ S). The samples were analyzed by SDS-PAGE and the radiolabeled proteins were detected by autoradiography.

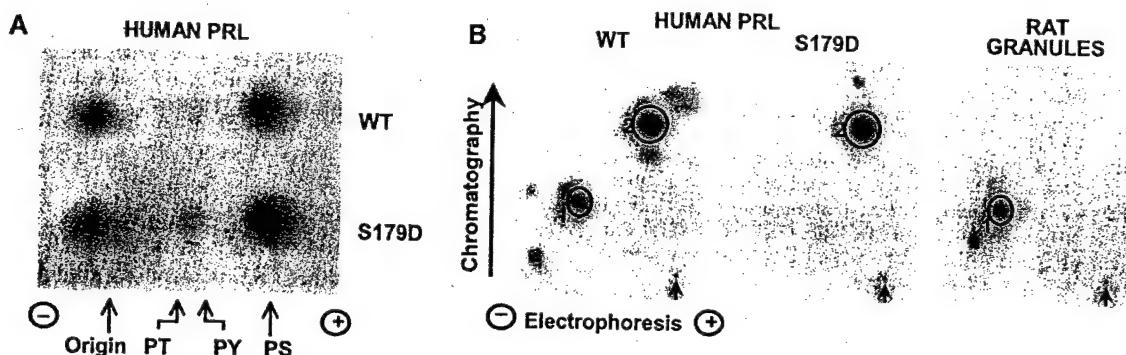


Fig. 2. Identification of a phosphorylation site for  $\gamma$ -PAK in PRL. A: Phosphoamino acid analysis of human PRL and S179D phosphorylated by  $\gamma$ -PAK. The positions of the phosphoamino acid standards are identified. B: Two-dimensional tryptic phosphopeptide mapping of wild-type PRL (left), S179D (middle) and PRL from rat granules (right) phosphorylated by exogenous  $\gamma$ -PAK. The  $^{32}$ P-labeled phosphoamino acids and phosphopeptides were detected using a phosphorimager system.

with 30–40% more phosphate incorporated into the wild-type protein, as compared to the mutant (Fig. 1B). Phosphoamino acid analysis showed only serine was phosphorylated in both wild-type PRL and S179D (Fig. 2A). Tryptic phosphopeptide mapping of wild-type PRL showed two major phosphopeptides. One of the phosphopeptides (spot 1) was missing in S179D, identifying serine 179 as a site phosphorylated by  $\gamma$ -PAK (Fig. 2B).

When solubilized PRL from rat granules was phosphorylated by exogenous  $\gamma$ -PAK, and the phosphorylation sites in PRL were analyzed by tryptic phosphopeptide mapping, only one tryptic phosphopeptide was observed (Fig. 2B). This phosphopeptide migrated at the same position as the tryptic phosphopeptide that contained phosphorylated Ser-179 (spot 1). Since the sequences of the tryptic phosphopeptide containing Ser-179 in human PRL and Ser-177 in rat PRL [34] are identical, we can conclude that  $\gamma$ -PAK phosphorylates Ser-177 in rat PRL.

### 3.2. $\gamma$ -PAK is present in PRL secretory granules

To determine whether the secretory granules contained

$\gamma$ -PAK, solubilized granule proteins were assayed for  $\gamma$ -PAK activity using histone 4. Histone 4 has been shown to be a specific substrate for PAKs; they are the only serine/threonine protein kinases reported to phosphorylate this substrate. Histone 4 was phosphorylated by a protein kinase in both bovine and rat secretory granules (Fig. 3A). Addition of GST- $\gamma$ -PAK resulted in increased phosphorylation of histone 4 and of rat PRL.

To determine the molecular weight of the histone 4 kinase, *in situ* phosphorylation of histone cast into SDS polyacrylamide gels was carried out using rat and bovine secretory granules, and purified recombinant  $\gamma$ -PAK. The protein kinase activity corresponded to the same molecular weight (58–60 kDa) as the recombinant  $\gamma$ -PAK standard (Fig. 3B). It also corresponded to the phosphoprotein bands in Fig. 3A which migrated at 58–60 kDa (see arrow to  $\gamma$ -PAK) in the absence of GST- $\gamma$ -PAK. The in-gel protein kinase activity was stimulated 1.5-fold when the solubilized proteins from bovine granules were preincubated with Cdc42(GTP $\gamma$ S) and ATP prior to analysis by SDS-PAGE and autoradiography (Fig. 3C); activation by Cdc42 is a characteristic feature of PAKs. Thus,

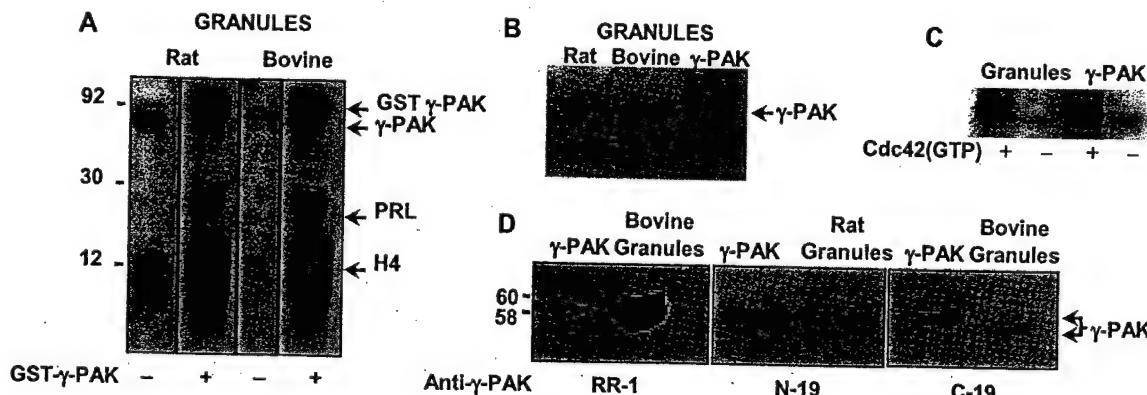


Fig. 3. Detection of  $\gamma$ -PAK in Secretory Granules. A: Proteins from secretory granules from rat (5  $\mu$ g) or bovine (10  $\mu$ g) were solubilized and assayed for  $\gamma$ -PAK activity using histone 4 as substrate. The samples were analyzed by SDS-PAGE on 15% gels, and the radiolabeled proteins were detected by autoradiography. B: Solubilized protein from rat granules (10  $\mu$ g) or from bovine granules (20  $\mu$ g), and recombinant  $\gamma$ -PAK (0.2  $\mu$ g), were analyzed by *in situ* phosphorylation of histone following SDS-PAGE on 10% polyacrylamide, as described in Section 2.  $\gamma$ -PAK activity was detected by autoradiography. C: Solubilized protein from bovine granules (20  $\mu$ g) and recombinant  $\gamma$ -PAK were incubated with Cdc42(GTP $\gamma$ S) and ATP prior to analysis by *in situ* phosphorylation of histone. D: Solubilized proteins from rat (10  $\mu$ g) and bovine (20  $\mu$ g) secretory granules were immunoblotted with antibodies N-19 and RR-1, which react specifically with the regulatory domain of  $\gamma$ -PAK, and C-19 which reacts with the highly conserved catalytic domain of the three PAK proteins. Detection was carried out with horseradish peroxidase-linked secondary antibodies and ECL reagents.

preactivation enhanced phosphorylation of histone in the gel, indicating both active and inactive forms of  $\gamma$ -PAK were present in the granule. Since  $\alpha$ - and  $\beta$ -PAK migrate at 68 and 65 kDa on SDS-PAGE, respectively, these forms of PAK were not present in any significant amounts in the granules.

Further confirmation of the identity of the protein kinase in PRL granules was provided by immunoblotting studies with antibodies specific for  $\gamma$ -PAK. A protein migrating at the same molecular weight as  $\gamma$ -PAK (58–60 kDa) was immunoreactive with antibodies specific for the N-terminus of  $\gamma$ -PAK, N-19 and RR-1. With N-19 antibody, multiple bands were detected, reflecting the multiple phosphorylated forms of  $\gamma$ -PAK [18]. C-19, prepared to the conserved catalytic domain of the PAKs, reacted only with protein migrating at 58 kDa, supporting the finding in Fig. 3C that  $\alpha$ - and  $\beta$ -PAK were not present in the granule preparation.

#### 4. Discussion

The biological activity of PRL has previously been shown to be regulated, in part, by the state of phosphorylation [30,42]. Non-phosphorylated PRL promotes growth, while phosphorylated PRL induces cytostasis. The major phosphorylation site in PRL is Ser-177/179 [33,34]. Here we show that  $\gamma$ -PAK is present in secretory granules and both endogenous and exogenous  $\gamma$ -PAK phosphorylate PRL on Ser-177/179.

Phosphorylation of PRL from secretory granules is more effective when the membrane is disrupted with Triton X-100, metal ions are chelated by EDTA and intermolecular bridges are disrupted by reducing agents; these aid in the formation of monomers from the multimeric semi-crystalline PRL [25,46]. PRL is also phosphorylated in secretory granule extracts by the endogenous protein kinase that has been identified as  $\gamma$ -PAK using a variety of methods. These include phosphorylation of the PAK-specific substrate histone 4, migration of the protein kinase activity at 58–60 kDa by an in-gel kinase assay, Western blotting with antibody specific for  $\gamma$ -PAK and activation of the endogenous protein kinase by Cdc42.

Recombinant human PRL is phosphorylated by  $\gamma$ -PAK at two sites as determined by tryptic phosphopeptide mapping. One of the phosphorylation sites is Ser-179 in phosphopeptide 1, as deduced from the disappearance of phosphopeptide 1 when the mutant S179D is phosphorylated by  $\gamma$ -PAK. The identity of phosphopeptide 2 is not known at this time. Only one major phosphopeptide is observed when PRL from rat secretory granules is phosphorylated by  $\gamma$ -PAK. The migration of this phosphopeptide is identical to the phosphopeptide that contains Ser-179 in human PRL; thus it can be concluded that rat PRL is phosphorylated specifically on Ser-177 by  $\gamma$ -PAK. Ser-177/179 is in the sequence RRDSHK that contains the recognition/phosphorylation determinant for  $\gamma$ -PAK [27]. Protein kinase A has been shown to phosphorylate this same site in rat PRL [33], although large amounts of the enzyme are needed to effect significant phosphorylation.

The reported sequence for  $\gamma$ -PAK contains no signal sequence that would automatically place it in the secretory pathway and eventually into secretory granules. Since the vast majority of  $\gamma$ -PAK is cytosolic [17,18], this is to be expected. However, it is unlikely that the  $\gamma$ -PAK activity observed in the granules is the result of contamination by cytoplasmic  $\gamma$ -PAK for three reasons: (1) preparation of the granules involves a

high salt wash with 1 M KCl; (2) previous studies have demonstrated that solubilization of the granule membrane, the material to which cytosolic  $\gamma$ -PAK could adhere during granule isolation, does not eliminate PRL kinase activity in the granules [43]; (3) there is a possibility that PAK in granules is N-glycosylated, since treatment with a deglycosylation enzyme, purified peptide- $N^{\alpha}$ -( $N$ -acetyl- $\beta$ -glucosaminyl)-asparagine amidase (PNGase F) [47], results in a decrease in molecular weight (data not shown). N-Glycosylation is a posttranslational modification which only occurs within the secretory pathway.

The question remains therefore as to how  $\gamma$ -PAK finds its way into the secretory granule to effect phosphorylation of PRL. There are several known mechanisms whereby proteins translated on free ribosomes can cross membranes after synthesis. Likely possibilities include the TAP protein mechanisms for cytosol to endoplasmic reticulum cisternal space import (for review see [48]) and the ABC-ATPase system, variants of which can move proteins from the cytosol into membrane-bound compartments in the cell, or can move them out of the cell through the plasma membrane [49]. Alternatively, it remains possible that a differentially spliced mRNA may be produced in the pituitary (as a minor species) which encodes a version with a signal sequence. Interestingly, protein kinase A and protein kinase C have been shown to be present in anterior pituitary granules [50], although they also do not contain a signal sequence.

The phosphorylated and non-phosphorylated forms of PRL have been shown to have different effects on cell growth. Phosphorylation at Ser-177/179 has a major effect on biological activity; it not only eliminates the growth-promoting qualities of PRL, but antagonizes the growth-promoting effect of unmodified PRL. For example, we recently showed that unmodified PRL can be an autocrine growth factor in human prostate cancer cells, and that the mimic of phosphorylated PRL (S179D) interrupts growth in vivo [51]. The data herein suggest that  $\gamma$ -PAK may retard growth in these and perhaps other cells via phosphorylation of PRL at Ser-177/179.  $\gamma$ -PAK has been shown to have cytostatic properties (as reviewed in [1]). It is interesting to note that apoptotic stimuli also activate  $\gamma$ -PAK and that S179D promotes apoptosis in the lung and thymus of exposed rat pups [52].

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# Different Biological Effects of Unmodified Prolactin and a Molecular Mimic of Phosphorylated Prolactin Involve Different Signaling Pathways<sup>†</sup>

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**ABSTRACT:** Previous work has shown that naturally phosphorylated prolactin antagonizes the growth-promoting activities of unmodified prolactin (U-PRL) and that this effect is duplicated by a molecular mimic, S179D PRL. At the same time, the S179D PRL is a superagonist with regard to expression of some PRL-regulated genes. We have asked whether the different activities of U-PRL and S179D PRL are the result of differential signaling. HC11 cells (a normal mouse mammary cell line) were grown to confluence, primed with hydrocortisone, and then exposed to the PRLs. A 15 min incubation of PRL-naïve cells led to substantial tyrosine phosphorylation of Jak 2 and Stat 5a by U-PRL and an essentially equivalent Jak 2 activation by S179D PRL. The latter, however, was accompanied by reduced tyrosine phosphorylation of Stat 5a. EMSA analysis using a Stat 5 binding site showed both PRLs to cause equivalent binding of nuclear proteins and that most of what bound was complexed through Stat 5a. Phosphoamino acid analysis of Stat 5 showed S179D PRL to double the amount of serine phosphorylation versus that seen with U-PRL. Analysis of the MAP kinase pathway showed U-PRL capable of activation of ERKs 1 and 2 but that signaling via ERKs 1 and 2 was greater with S179D PRL. A 7-day incubation in either PRL increased  $\beta$ -casein mRNA levels, but S179D PRL caused a 2-fold increase over that seen with U-PRL. The increase, over that seen with U-PRL, was blocked by the MAP kinase inhibitor, PD98059. After 7 days of treatment with S179D PRL, expression of the short PRL receptor was doubled, and signaling showed a greater dependence on the MAP kinase pathway (2.9-fold increase in ERK 1 and 2 activation). We conclude that although both PRLs use both pathways to some extent, U-PRL signals primarily through Jak 2–Stat 5 whereas S179D PRL signals primarily through the MAP kinase pathway especially after prolonged exposure. This is the first demonstration of differential involvement of signaling pathways by different forms of PRL.

Work from this laboratory has recently described the differential effects of recombinant unmodified prolactin (U-PRL)<sup>1</sup> and a molecular mimic of phosphorylated PRL (S179D PRL) on the development of the adult rat mammary gland (1). Thus, U-PRL was shown to promote ductal growth

and the growth of alveoli, and S179D PRL was shown to inhibit ductal and alveolar growth and yet to promote  $\beta$ -casein gene expression. The growth and anti-growth effects of these PRLs in the rodent mammary gland have recently been duplicated in another laboratory (C. Ormandy, Garvan Institute, Sydney, Australia, personal communication). Given the differential effects of these two PRLs in the mammary gland, it seemed likely that their interaction with PRL receptors resulted in the use of differential signaling pathways within mammary epithelial cells. Differential intracellular signaling of these two PRLs has previously been demonstrated in the Nb2 lymphoma system (2). However, the PRL receptor of Nb2 cells is unique (3), and analysis of signaling via this unique receptor may not provide accurate insight into signaling in other tissues. All normal rodent tissues express both a long and a short form(s) of the PRL receptor (4). The ratio of long to short receptor varies from tissue to tissue and has been shown to vary with physiological state in some tissues (4). The significance of this differential expression is still unclear. Both forms of the normal PRL receptor are identical in their extracellular domains and differ in their cytoplasmic domains (5). They have both been shown to activate Jak 2 (6), but only the long form of the receptor activates Stat 5 as a result (6, 7). In addition, the long and short forms of the receptor also activate the MAP kinase

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<sup>1</sup> Abbreviations: PRL, prolactin; U-PRL, unmodified prolactin; S179D PRL, mimic of phosphorylated prolactin in which serine 179 is replaced by an aspartate residue; EMSA, electromobility shift assay; MAP kinase, mitogen-activated protein kinase; ERK, extracellular signal regulated kinase; Jak 2; Janus kinase 2; Stat 5, signal transducer and activator of transcription 5; NIDDK, National Institute for Diabetes, Digestive and Kidney Diseases; NICHD, National Institute for Child Health and Human Development; USDA, United States Department of Agriculture; GIBCO, Grand Island Biological Company; RPMI, Roswell Park Memorial Institute; MALDI, matrix-assisted laser desorption ionization; PD98059, 2-(2-amino-3-methoxyphenyl)-4*H*-1-benzopyran-4-one; Tris/TRIZMA, tris(hydroxymethyl)aminomethane; PMSF, phenylmethanesulfonyl fluoride; EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulfate; BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle's medium; PVDF, polyvinylidene fluoride; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*'-2-ethanesulfonic acid; DTT, dithiothreitol; GAS,  $\gamma$  interferon activating sequence; TBE, Tris-borate-EDTA; SSC, sodium citrate/sodium chloride; UV, ultraviolet; PCR, polymerase chain reaction.

pathway via ras and raf (8, 9). These appear to be the main signaling pathways, although a large number of other signaling molecules have been shown to be activated by PRL in a variety of tissues (reviewed in ref 10).

In this study, we have used a normal mouse mammary cell line to investigate the relative use of the Jak 2–Stat 5 pathway and the MAP kinase pathway by each form of PRL. Although both PRLs use both pathways to some extent, the results demonstrate that U-PRL predominantly uses Jak 2–Stat 5a, whereas S179D PRL predominantly uses MAP kinase.

## EXPERIMENTAL PROCEDURES

**Mass Spectrometry.** The phosphorylation status of normal human PRL extracted from pituitaries (standard PRL) was assessed by mass spectrometry. The human PRL was in part a gift from Dr. A. Parlow (Harbor-UCLA, Torrance, CA) and in part provided through the auspices of the Hormone and Pituitary Program of the NIDDK, NICHD, and USDA. The PRL (AFP3855A, B3) was dissolved in water and washed to reduce ion contamination by three exchanges of water in a Microcon YM-10 (Millipore Corp., Bedford, MA) with a 10 kDa molecular mass cutoff. The washed material was completely dissolved in water containing 0.1% trifluoroacetic acid, and aliquots were subjected to MALDI (matrix-assisted laser desorption ionization) using sinapinic acid as the matrix. The choice of matrix is crucial to appropriate resolution of the phosphorylated forms. Accuracy in this molecular mass range is  $\pm 0.1\%$ .

**Recombinant PRLs.** The recombinant human PRLs were expressed in *Escherichia coli* to avoid posttranslational modifications and were produced and characterized as previously described (11). Since both PRLs were expressed at similar levels and were isolated and folded in tandem, they served as controls for one another for any potential non-PRL contaminants. Serine 177 in rat PRL is the major site of phosphorylation (12) and is equivalent to serine 179 in human PRL (Swiss Protein Database). Serine 179 of human PRL has also been shown to be phosphorylated by the intragranular kinase of rat pituitary secretory granules (13). Mutation of S179 to an aspartate residue produced a molecular mimic of monophosphorylated PRL. An aspartate residue mimics a phosphoserine by approximate size of the side chain and by carrying a negative charge. This kind of mimicry is now commonplace in enzymology where both functional and structural studies show equivalency between the natural phospho form and the mimic (e.g., ref 14).

**HC11 Cells.** HC11 cells, which are normal mouse mammary epithelial cells, were generously provided by Dr. Nancy Hynes (Friedrich Meischer Institute, Basel, Switzerland) through Dr. Margaret Neville (University of Colorado, Denver, CO). They were routinely cultured in RPMI 1640 with 10% fetal bovine serum (GIBCO-BRL, Grand Island, NY), 5  $\mu$ g/mL insulin (Sigma Chemical Co., St. Louis, MO), and 10 ng/mL EGF (GIBCO-BRL). When the cells had been confluent for 2 days, the medium was changed to “priming medium” (RPMI 1640, 10% charcoal-stripped horse serum, 10  $\mu$ g/mL insulin, and 1  $\mu$ g/mL hydrocortisone) for 1–2 days. In the first experiments, standard PRL, U-PRL, or S179D PRL was then added to give a final concentration of 5  $\mu$ g/mL, and the cells were lysed after 15 min. In the second

set of experiments, 1  $\mu$ g/mL U-PRL or S179D PRL was used. In the third set, different doses of S179D PRL were titrated against 100 ng/mL U-PRL. The priming protocol was provided by Peggy Neville and was based on one designed by Taverna et al. (15). Of special note for this study was analysis of signaling with or without only the PRLs. In other words, unlike studies published by other laboratories (e.g., ref 16), all three lactogenic hormones were not added simultaneously. Instead, the cells had been exposed to insulin throughout and to hydrocortisone for 1–2 days prior to the addition of PRL.

HC11 cells are the only cell line that responds to PRL by increasing  $\beta$ -casein gene expression. This is therefore the only cell line in which one can analyze natural regulation. Even in this cell line, however, the level of  $\beta$ -casein expression is relatively low, and others have demonstrated that there is an increasing response to PRL treatment for as much as 5 days (17). Since our goal was to analyze mRNA levels by Northern blot and to assess differential total stimulation between the two PRLs, we utilized a 7-day stimulation period.

When  $\beta$ -casein gene expression was analyzed, cells were incubated in a 1  $\mu$ g/mL amount of each PRL for 7 days with a change of medium each day. Signaling in 7-day PRL-incubated cells was assessed by removal of PRL for 2 h prior to reexposure. As for initial exposure, reexposure was for 15 min. In some experiments, the MAP kinase inhibitor, PD98059 (25  $\mu$ M), was included in the 7-day PRL incubation. This concentration of PD98059 has been shown to decrease PRL-stimulated MAP kinase activity in HC11 cells by more than 90% (16).

After PRL exposure, cells were rinsed with 0.01 M phosphate-buffered saline, pH 7.4, and were then scraped from the dish into 20 mM Tris, pH 7.4, containing 150 mM NaCl, 10 mM each of sodium pyrophosphate, sodium fluoride, and sodium vanadate, 10  $\mu$ g/mL each of aprotinin, leupeptin, and pepstatin, 1 mM PMSF, 0.02% sodium azide, 1 mM EDTA, and 1% Triton X-100. After a 1 h rotation at 4 °C, lysates were cleared by centrifugation at 13000g for 10 min, and the supernatants were saved for immunoprecipitation.

**Immunoprecipitation.** Lysates containing 4 mg of protein were used for immunoprecipitation. Antibodies were as follows: polyclonal anti-Jak 2 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), polyclonal anti-Stat 5b (Zymed Laboratories, Inc., San Francisco, CA), polyclonal anti-pan Stat 5 and anti-Stat 5a (Santa Cruz Biotechnology), and polyclonal anti-MAP kinase (Promega, Madison, WI). Four micrograms of purified antibody was added to 1 mL of lysate and incubated for 2 h overnight (overnight used for anti-MAP kinase, but 2 h or overnight made no difference to the Stat 5 results) at 4 °C. Antigen–antibody complexes were precipitated by addition of 30  $\mu$ L of washed protein G–Sepharose slurry (Amersham Pharmacia Biotech, Piscataway, NJ) and incubation for 2 h at 4 °C. The pellets were washed three times in lysis buffer and then placed in reducing SDS sample buffer at 95 °C for 10 min before being loaded on a 7.5% polyacrylamide gel. For Figure 6, whole cell lysates were subjected to electrophoresis.

**Western Blot.** After protein transfer to nitrocellulose membranes in 48 mM Trizma, 30 mM glycine, 0.1% SDS, and 10% methanol (pH 8.3), membranes were blocked with

10% nonfat milk in wash buffer (20 mM Tris, pH 7.4, 150 mM NaCl, 1% BSA, 0.1% Tween 20). Blocked membranes were probed with anti-phosphotyrosine (Upstate Biotechnology, Inc., Lake Placid, NY) diluted 1:2000 in wash buffer or with anti-phospho MAP kinase (Promega) diluted 1:5000 for 2 h at room temperature. After being washed three times for 15 min, the blot was further incubated in goat anti-mouse conjugated to horseradish peroxidase (Sigma) at 1:2000 or goat anti-rabbit conjugated to horseradish peroxidase (Sigma), as appropriate, for 1 h at room temperature. After five washes, horseradish peroxidase positive bands were detected with ECL reagent (Amersham Biosciences, Piscataway, NJ) followed by autoradiography. Blots were subsequently stripped for 1 h at 70 °C in 100 mM  $\beta$ -mercaptoethanol and 2% SDS in 62.5 mM Tris, pH 6.8. After further blocking and washing, membranes were reprobed with anti-Jak 2, anti-Stat 5a, anti-Stat 5b, or anti-MAP kinase diluted 1:1000 in wash buffer, and either goat anti-rabbit or goat anti-mouse peroxidase conjugated antibodies were used, as appropriate.

Controls included tests of the second antibodies alone for each of the immunoprecipitating antibodies. Proteins were identified by molecular mass in reference to coelectrophoresed colored standards (Bio-Rad, Hercules, CA) and by overlay of the autoradiograms derived from sequential blots. Each blot analyzing phosphotyrosine included a positive control, which also served as a control for thorough stripping of membranes. For each blot, several exposures were used to ensure that film development was in the linear range.

**Phosphoamino Acid Analysis.** After 2 days in priming medium, the medium was changed to phosphate-free DMEM (GIBCO-BRL) containing 1 mCi/mL  $H_3^{32}PO_4$  (ICN Radiochemicals, Irvine, CA) for 4 h prior to a 15 min incubation in the PRLs. At the end of the 15 min incubation, the cells were lysed, and Stat 5 was immunoprecipitated as above using the anti-Stat 5 antibody. The immunoprecipitate was resolved into its components by reducing SDS gel electrophoresis, and the proteins were transferred to PVDF membranes and then subjected to autoradiography. The Stat 5 band, identified by reference to molecular weight markers, was excised and then hydrolyzed in 6 N HCl (Pierce Chemical Co., Rockford, IL) for 1 h at 110 °C under vacuum. Hydrolysates were dried in a SpeedVac and then redissolved in water. Samples were spotted on thin-layer chromatography films (EM Science, Gibbstown, NJ) and electrophoresed at 450 V for 3 h at 4 °C in 0.5% pyridine containing 0.5 mM EDTA, pH 3.9. After drying and visualization of co-run standards with ninhydrin (0.3% in butanol), the phosphoamino acids were detected by autoradiography and quantified by densitometry.

**Electromobility Shift Assay.** To obtain a nuclear extract, cells were first scraped off the dish into hypotonic buffer [20 mM Tris, pH 7.4, containing 10 mM NaCl, 1 mM MgCl<sub>2</sub>, 10  $\mu$ g/mL each of aprotinin, leupeptin, and pepstatin (Sigma), 1 mM PMSF (Sigma), 10 mM NaF, and 1 mM Na<sub>3</sub>VO<sub>4</sub>] and allowed to swell on ice. The cells were broken by two passages through a 27 gauge needle, and nuclei were then pelleted by centrifugation. The nuclear pellet was then placed in hypertonic buffer (20 mM Hepes, pH 7.5, containing 420 mM KCl, 1.5 mM MgCl<sub>2</sub>, 0.5 mM EDTA, 20% glycerol, 10  $\mu$ g/mL each of aprotinin, leupeptin, and pepstatin, 1 mM PMSF, 10 mM NaF, and 1 mM Na<sub>3</sub>VO<sub>4</sub>) for 30 min on ice with occasional mixing.

After centrifugation at 12000g for 15 min, the supernatants were diluted with equal volumes of the hypotonic buffer to lower the salt concentration. Two micrograms of nuclear extract protein was used in each binding reaction, which was carried out in 10 mM Hepes, pH 7.4, containing 50 mM KCl, 5 mM MgCl<sub>2</sub>, 10% glycerol, and 5 mM DTT. One microgram of antibody or 100 $\times$  unlabeled oligonucleotide or an equivalent volume was added for 15 min followed by incubation for 45 min at 37 °C with radiolabeled oligonucleotide. The oligonucleotide equivalent to the most proximal GAS site on mouse  $\beta$ -casein (5'-CACGTAGACTCTTG-GAATTGA-3') was annealed to the complementary sequence and radiolabeled with T4 kinase (GIBCO-BRL, Grand Island, NY) according to the manufacturer's instructions. At the end of the binding reaction, the mixture was analyzed on a 6% polyacrylamide gel (prerun for 30 min at 120 V) in 0.25  $\times$  TBE buffer (22.5 mM Tris, 22.5 mM boric acid, 0.5 mM EDTA, pH 8) for 1.5 h at 200 V. Dried gels were exposed for autoradiographic analysis.

**Northern Blot Analysis for the Expression of  $\beta$ -Casein and the Long and Short Forms of the PRL Receptor mRNA.** Total RNA, isolated from HC11 cells using the Trizol RNA reagent (GIBCO-BRL), was treated with DNase I (GIBCO-BRL). Equal amounts of RNA (10  $\mu$ g) from control and test samples were loaded on a 1.0% agarose-formaldehyde gel. The gels were run at 60 V for 3–5 h. The RNA was blotted onto nylon filters (Micron Separations, Inc., Westboro, MA) by capillary transfer with 10  $\times$  SSC and fixed by UV cross-linking. The 201 bp mouse  $\beta$ -casein cDNA probe for hybridization was made by PCR. The primers were 5'-CCC GTC CCA CAA AAC ATC C-3' (forward) and 5'-ATT AGC AAG ACT GGC AAG GCT G-3' (reverse). A 399 bp cDNA probe which recognizes both the long and short forms of the receptor was made by PCR. The primers were 5'-CCC ACC CAC CAT AAC TGA TG-3' (forward) and 5'-TCC AGC AGA TGG GTA TCA AAT C-3' (reverse). Hybridization gave a band at  $\sim$ 10 kb and a band at  $\sim$ 8 kb for the long and short receptor message, respectively. A 245 bp specific probe for the short receptor was a gift from Paul Kelly's laboratory (INSERM, Paris, France). Probes were labeled with 50  $\mu$ Ci of [ $\alpha$ -<sup>32</sup>P]dCTP (ICN) using a DECA Prime II DNA labeling kit (Ambion, Austin, TX). The labeled probes were separated by ProbeQuant G-50 microcolumns (Amersham Pharmacia Biotechnology). After 2 h of prehybridization at 65 °C with the hybridization solution (25 M Na<sub>2</sub>HPO<sub>4</sub>, pH 7.2, 1 mM EDTA, pH 8.0, 7% SDS), hybridizations were carried out at 65 °C for 16–24 h. The filters were then washed in alternating solutions of 20 mM Na<sub>2</sub>HPO<sub>4</sub>, pH 7.2, 1 mM EDTA, and 5% SDS and of 20 mM Na<sub>2</sub>HPO<sub>4</sub>, pH 7.2, 1 mM EDTA, and 1% SDS for a total of three times in each. Filters were exposed to Fuji medical X-ray film (Fuji Medical Systems, Inc., Stamford, CT) for 1–7 days at  $\sim$ 70 °C. Probe stripping was performed by heating the nylon filter at 95 °C for 10–30 min in a solution of 10 mM Tris, pH 8.0, 1 mM EDTA, and 1% SDS. A mouse 18S rRNA cDNA fragment (DECA template-18S-mouse, 1212 bp) (Ambion, Austin, TX) was used to normalize for errors in RNA loading and transfer. A Kodak 1D image analysis system was used for quantification (Eastman Kodak Co., Rochester, NY). Several exposures were used for each autoradiogram to ensure that film development was in the linear range.

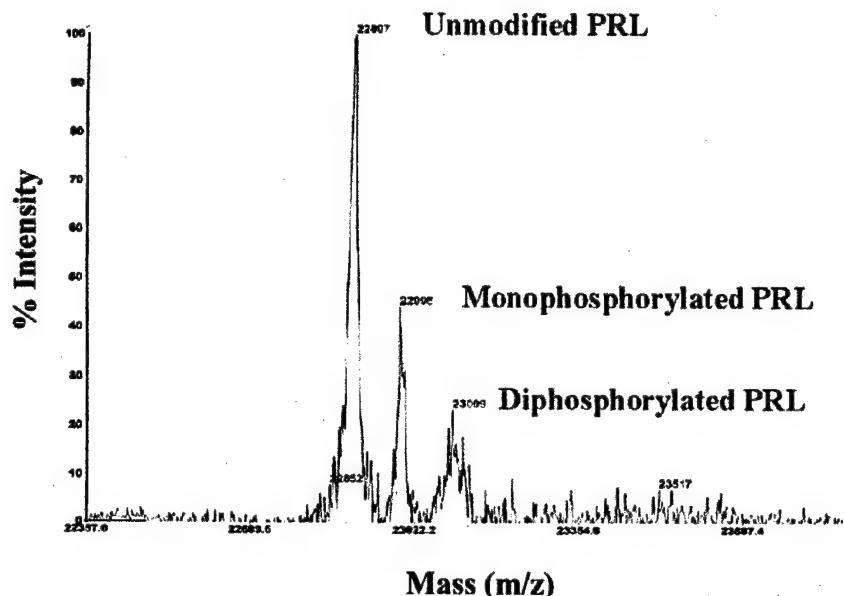


FIGURE 1: Mass spectrometric analysis of NIDDK standard human pituitary PRL. PRL was dissolved in 0.1% trifluoroacetic acid and was subjected to matrix-assisted laser desorption ionization using sinapinic acid as the matrix. The ordinate is in relative units. The numbers next to the peaks are the masses of those peaks in daltons. The accuracy in this molecular mass range is  $\pm 0.1\%$ . The main peak at 22897 is unmodified PRL, the peak at 22996 is monophosphorylated PRL, and the peak at 23099 is diphosphorylated PRL.

**Statistical Analyses.** Data were analyzed by analysis of variance and posttests for comparing specific groups, using Bonferroni corrections for multiple comparisons against a single group.

## RESULTS

Mass spectrometry of standard human pituitary PRL demonstrated the presence of mono- and diphosphorylated PRL within the mixture (Figure 1). The first peak at a mass of 22897 Da is U-PRL, which has a calculated mass of 22897.75 Da. It constitutes 62% of the mixture. The second peak at 22996 Da is PRL with one phosphate and one sodium exchanged for a hydrogen. It constitutes 19% of the mixture. The third peak at 23099 Da is PRL with two phosphates and two sodium–hydrogen exchanges. It also constitutes 19% of the mixture. The proportions of each form of PRL were in the range reported previously for rodent PRL (18, 19). Signaling from standard PRL therefore represents a physiological, mixed response to U-PRL and phospho-PRL. This preparation of standard PRL, however, only represents one ratio of U-PRL to phospho-PRL, and the amount phosphorylated varies with physiological status (18, 19) and varies from preparation to preparation distributed as standard.

To clearly differentiate the intracellular signals of U-PRL and P-PRL, we used a recombinant human version of U-PRL and a recombinant molecular mimic of human P-PRL, S179D PRL. In this way we could avoid the potential dephosphorylation of P-PRL to U-PRL. In addition, we compared signaling generated by these two recombinant PRLs to that produced by the standard PRL preparation. In this way, we could compare our results with others in the literature.

As shown in Figure 2, primed HC11 cells show some activation of Jak 2 without the addition of PRL. This occurred even when the cells were allowed to rest prior to and during the 15 min incubation. Addition of standard PRL, recombinant U-PRL, or S179D PRL resulted in a similar degree

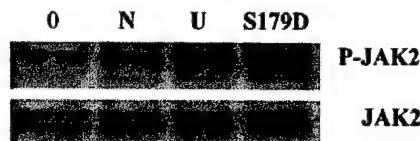


FIGURE 2: Jak 2 activation by U-PRL, S179D PRL, and standard pituitary PRL. HC11 cells were exposed to 5  $\mu$ g/mL PRLs for 15 min. Immunoprecipitation was with anti-Jak 2. The upper panel was blotted with anti-phosphotyrosine and then stripped and reprobed with anti-Jak 2 to produce the lower panel. Key: 0, no added PRL; N, addition of NIDDK standard human pituitary PRL; U, unmodified recombinant human PRL; S179D, recombinant S179D human PRL. Note that each PRL activates Jak 2 and that the degree of activation is similar, although the background in the S179D PRL-treated lane is higher. This blot is representative of three separate experiments.

of additional Jak 2 activation with, if anything, a larger increase in response to S179D PRL (upper panel blotted with anti-phosphotyrosine). Equal loading of the lanes is demonstrated in the bottom panel blotted with anti-Jak 2. Analysis of Stat 5 tyrosine phosphorylation, however, showed very different responses to the different PRLs. Standard human PRL (which is mostly U-PRL) and U-PRL strongly tyrosine phosphorylated Stat 5a while S179D PRL showed a more modest increase over the control level (Figure 3). By contrast, none of the PRLs was a strong activator of Stat 5b tyrosine phosphorylation in these cells (data not shown).

Following exposure of the cells to each PRL, EMSA analysis using the  $\beta$ -casein GAS site demonstrated that each PRL promoted the nuclear translocation of proteins which formed very similar protein–oligonucleotide complexes, resolving as two to three bands (Figure 4). Competition with anti-Stat 5a and anti-Stat 5b antibodies demonstrated that the majority of the complexes contained Stat 5a, regardless of the PRL type. The absence of the complexes with no PRL and in the presence of competing, unlabeled oligonucleotide shows the specificity of the binding. Excess, radiolabeled

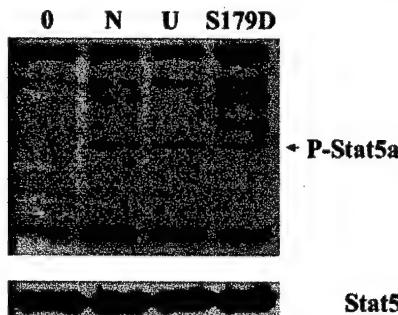


FIGURE 3: Stat 5a activation in response to U-PRL, S179D PRL, and standard pituitary PRL. HC11 cells were exposed to 5  $\mu$ g/mL PRLs for 15 min. Immunoprecipitation was with anti-Stat 5a. The upper panel was blotted with anti-phosphotyrosine and then stripped and reprobed to produce the lower panel blotted with anti-Stat 5a. Key: 0, no added PRL; N, addition of NIDDK standard human pituitary prolactin; U, addition of unmodified recombinant human PRL; S179D, addition of recombinant human S179D PRL. Note the superior tyrosine phosphorylation by NIDDK PRL and U-PRL and the weaker activation by S179D PRL. This blot is representative of eight separate experiments.

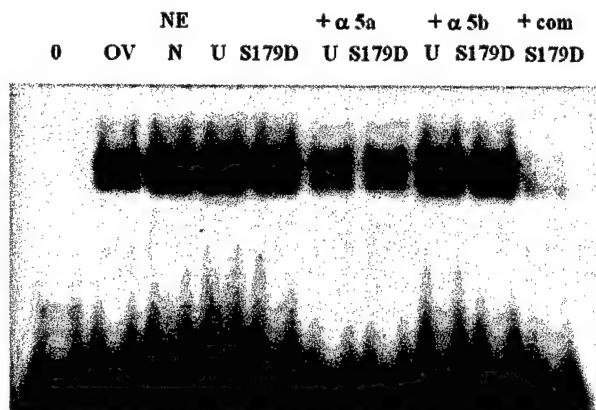


FIGURE 4: EMSA analysis of the  $\beta$ -casein GAS site following stimulation with U-PRL, S179D PRL, and standard pituitary PRL. After stimulation of the cells with each PRL (5  $\mu$ g/mL, 15 min), nuclear extract proteins (NE) were incubated with radiolabeled GAS site double-stranded oligonucleotide with or without competition by anti-Stat 5a ( $\alpha$  5a), anti-Stat 5b ( $\alpha$  5b) or 100-fold unlabeled oligonucleotide (com). Key: 0, no added PRL; OV, addition of ovine PRL; N, addition of NIDDK standard human pituitary PRL; U, addition of unmodified recombinant human PRL; S179D, addition of recombinant human S179D PRL. Note the very similar complexes produced by NIDDK PRL, U-PRL, and S179D PRL and that formation of complexes in the latter two (only two tested) could be competed for by anti-Stat 5a but not anti-Stat 5b. This autoradiogram is representative of four separate experiments.

oligonucleotide also ensured comparability on a relative quantitative basis.

Phosphoamino acid analysis of immunoprecipitated Stat 5 following phosphate radiolabeling of the cells showed incubation with S179D PRL to result in less tyrosine phosphorylation overall, but in two to three times the level of serine phosphorylation versus that seen with U-PRL (Figure 5). As has been observed by others, there was a constitutive level of serine phosphorylation in cells that had not been exposed to PRL (16, 20), and this level was not increased by U-PRL. Standard PRL gave a result intermediate between the other two PRLs, a result reflective of it being a mixture of U-PRL and P-PRL. The results were normalized to the total radiolabeled phosphate in each lane to correct

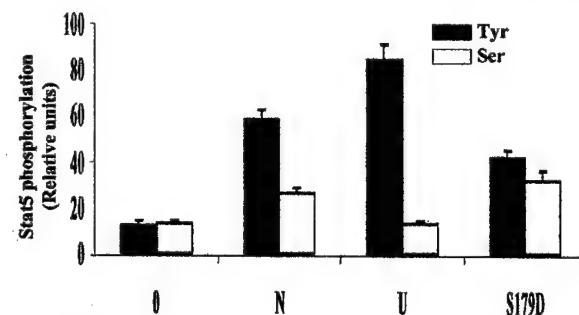


FIGURE 5: Tyrosine and serine phosphorylation of Stat 5 in response to U-PRL, S179D PRL, and standard pituitary PRL. HC11 cells were incubated in 5  $\mu$ g/mL PRLs for 15 min, lysed, and subjected to immunoprecipitation with anti-Stat 5. Hydrolysis of the gel-purified Stat 5 was followed by phosphoamino acid analysis. The bars show the means  $\pm$  SE of three separate phosphoamino acid analyses from one radiolabeling experiment and are expressed as relative densitometric units normalized to total phosphate in the hydrolysate to correct for loading. Key: Tyr, phosphotyrosine; Ser, phosphoserine; 0, no added PRL; N, addition of NIDDK standard human PRL; U, addition of recombinant human unmodified PRL; S179D, addition of recombinant human S179D PRL.

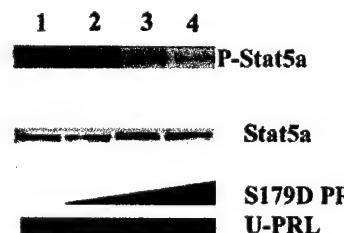


FIGURE 6: Competition between U-PRL and S179D PRL for Stat 5 tyrosine phosphorylation. All cells were incubated in 100 ng/mL U-PRL. Cells in lanes 2, 3, and 4 were additionally incubated in 10, 100, and 1000 ng/mL S179D PRL, respectively. This experiment was conducted without immunoprecipitation of Stat 5a. The upper panel showing phosphotyrosine reactivity was exposed for 5 min. After being stripped and reprobed with anti-Stat 5a, the lower panel was exposed for 20 s. These blots are representative of three separate experiments.

for loading of the film. Both NIDDK PRL and S179D PRL showed a higher amount of free phosphate, suggesting a reduced stability of the phosphoamino acids in these samples. Without this normalization, the differences between the U-PRL and S179D PRL samples would have been larger.

Given the differential effects of U-PRL and S179D PRL on tyrosine phosphorylation of Stat 5a, we compared the titration of one form of PRL against the other. To ensure that the observed differences in tyrosine phosphorylation were not an artifact of preferential immunoprecipitation of serine-phosphorylated Stat 5, this experiment was conducted without immunoprecipitation. Figure 6 illustrates that increasing concentrations of S179D PRL progressively decrease Stat 5a tyrosine phosphorylation in response to U-PRL. Further, this experiment demonstrates that immunoprecipitation was not selecting for subpopulations of Stat 5a.

Because previous work had demonstrated that S179D PRL had a superior ability to stimulate  $\beta$ -casein gene expression (1) and this was clearly not due to superior tyrosine phosphorylation of Stat 5a or Stat 5b, we investigated the effect of each PRL on the MAP kinase signaling pathway. In these experiments, the two recombinant PRLs were tested for their ability to activate ERKs 1 and 2, and this was concurrently analyzed with activation of Stat 5a. As shown

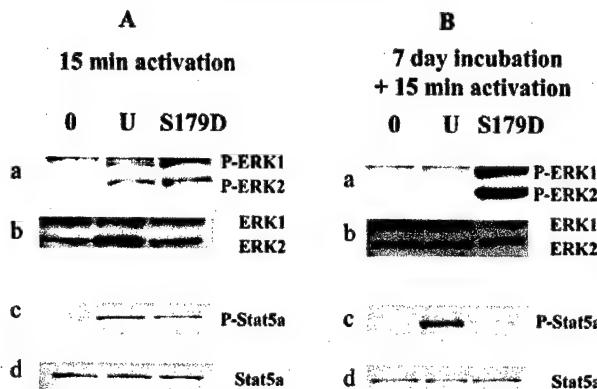


FIGURE 7: Activation of ERK 1, ERK 2, and Stat 5a in response to U-PRL and S179D PRL, both before (panel A) and after (panel B) a 7-day incubation in those PRLs. The cells were incubated for 15 min for panel A in 5  $\mu$ g/mL PRLs. For panel B, the cells were incubated in the PRLs at 1  $\mu$ g/mL for 7 days; the PRLs were withdrawn for 2 h and then reappplied for 15 min (5  $\mu$ g/mL). The upper panels (a) show the result of immunoprecipitation with anti-total ERK and blotting with anti-active ERK. Below them is the same blot stripped and reprobed with anti-total ERK (b). The third panel down shows the result of immunoprecipitation with anti-Stat 5a and blotting with anti-phosphotyrosine (c), and the lowest panel shows the result of stripping and reprobing with anti-Stat 5a (d). In each case, the A panels and B panels were exposed to the same film so that direct comparisons could be made before and after the 7-day incubation. Note the greater activation of ERKs 1 and 2 by S179D PRL and the greater activation of Stat 5a by U-PRL. Also note the upregulation of ERK signaling after the 7-day incubation in S179D PRL and downregulation of Stat 5a signaling. These blots are representative of five separate experiments.

In Figure 7Aa, S179D PRL caused a substantial activation of both ERKs, while the response to U-PRL was weaker. This weaker response to U-PRL is emphasized when one notes the heavier loading of total ERKs shown in Figure 7Ab. At the same time in these experiments, the initial differential responses in terms of Stat 5a were reproduced. In other words, S179D PRL was less effective than U-PRL in causing Stat 5a tyrosine phosphorylation.

To determine whether activation of ERKs 1 and 2 was related to superior  $\beta$ -casein gene expression, we utilized the inhibitor PD98059. As before (1), incubation of HC11 cells in either PRL for 7 days resulted in upregulation of endogenous  $\beta$ -casein gene expression (Figure 8). U-PRL doubled the control level (compare bars 1 and 3), while S179D PRL quadrupled the control level (compare bars 1 and 5). Addition of the MAP kinase inhibitor PD98059 inhibited the S179D PRL response (compare bars 5 and 6) while it had no effect on the basal level of expression (compare bars 1 and 2). Importantly, PD98059 also had no effect on  $\beta$ -casein gene expression stimulated by U-PRL (compare bars 3 and 4). Thus MAP kinase activation is linked to the superior  $\beta$ -casein expression seen in response to S179D PRL but is not linked to U-PRL-stimulated  $\beta$ -casein gene expression.

Because one cannot extrapolate signaling after a 15 min exposure to what has happened during the 7-day incubation period required to effectively analyze endogenous gene expression by Northern blot, we reanalyzed signaling at the end of the 7-day period in the same set of cells. Reanalysis of signaling after the 7-day exposure to each of the two PRLs showed a 2.9-fold upregulation of ERK 1 and 2 activation

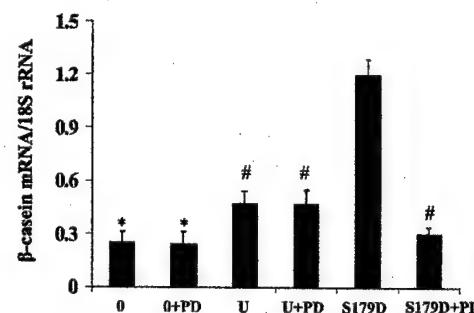


FIGURE 8: Expression of  $\beta$ -casein mRNA in response to U-PRL and S179D PRL in the presence and absence of the MAP kinase inhibitor, PD98059. HC11 cells were incubated in the PRLs for 7 days in the absence or presence of PD98059 (PD). The PRLs were given at 5  $\mu$ g/mL, and the PD98059 was given at 25  $\mu$ M. Key: 0, no addition of PRL; 0 + PD, PD alone; U, addition of unmodified recombinant human PRL; U + PD, addition of unmodified recombinant human PRL plus PD; S179D, addition of recombinant human S179D PRL; S179D + PD, addition of recombinant S179D human PRL plus PD. The data are derived from Northern blots, normalized to 18S rRNA, and are expressed as the mean  $\pm$  SE of three separate experiments. \* =  $p < 0.01$ ; # =  $p < 0.05$  versus S179D PRL. Note that PD had no effect on U-PRL-stimulated expression while it inhibited the additional stimulation brought about by S179D PRL.

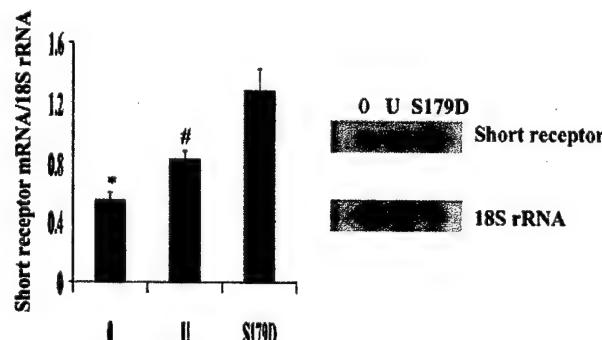


FIGURE 9: Expression of the short PRL receptor in response to a 7-day incubation in 1  $\mu$ g/mL U-PRL or S179D PRL. Key: 0, no added PRL; U, addition of recombinant unmodified human PRL; S179D, addition of recombinant human S179D PRL. The data are derived from Northern blots using a probe specific for the short receptor, normalized to 18S rRNA, and are presented as the mean  $\pm$  SE from five separate experiments. This probe does not distinguish among the three short forms of the receptor. \* =  $p < 0.01$ ; # =  $p < 0.05$  versus S179D PRL. Note the doubling in the expression of the short receptor in response to S179D PRL.

in the S179D PRL-treated cells (compare Figure 7Aa with Figure 7Ba) with concomitant downregulation of Stat 5a activation (compare Figure 7Ac with Figure 7Bc). For each antibody, panels A and B were produced on the same autoradiogram and hence can be directly compared. U-PRL, by contrast, upregulated signaling through Stat 5a (compare Figure 7Ac with Figure 7Bc). Concomitant analysis of long and short PRL receptor expression after the 7-day incubation showed a doubling of the expression of the short receptor as a result of S179D PRL treatment and a smaller increase with U-PRL (Figure 9). Expression of the long receptor changed very little (data not shown).

When the short to long PRL receptor ratio is plotted against  $\beta$ -casein gene expression, one can appreciate that an increasing ratio correlates with increasing  $\beta$ -casein gene expression (Figure 10), suggesting a link between increased

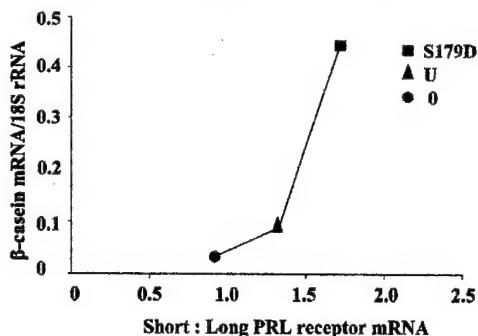


FIGURE 10: Relationship between  $\beta$ -casein gene expression and the ratio of short to long PRL receptor expressed. Cells were incubated in the PRLs at  $1 \mu\text{g/mL}$  for 7 days. The data are derived from five separate experiments using the probe which recognizes both long and short receptor mRNA. Quantification of the long and short receptor was therefore on the same autoradiogram. Note the increasing  $\beta$ -casein expression with increasing short to long receptor ratio.

short receptor expression, increased use of the MAP kinase pathway, and increased  $\beta$ -casein expression.

## DISCUSSION

Previous experiments have shown very different biological activities of U-PRL and S179D PRL in the adult rodent mammary gland (1). Thus U-PRL promotes growth, while S179D PRL inhibits growth and yet promotes  $\beta$ -casein gene expression. It seemed likely therefore that these two PRLs initiated different signaling pathways in mammary epithelial cells.

S179D PRL is a molecular mimic of human P-PRL. P-PRL has been shown to be a proportion of pituitary PRL in rats, cows, sheep, turkeys, and chickens (21–23). Previous work had indirectly demonstrated that human PRL was phosphorylated by showing an increase in Nb2 proliferative activity of standard human pituitary PRL after treatment with acid phosphatase (11). The current paper shows more directly by mass spectrometry that a proportion of human pituitary PRL is phosphorylated. In experimental animals where this can be analyzed, studies have shown physiological regulation of the proportion phosphorylated (18, 19). Thus, in the second two-thirds of rodent pregnancy, there is a high proportion of U-PRL, but P-PRL increases prior to parturition (19; unpublished data). In addition, mammary epithelial cells are exposed via apically oriented receptors (24) to high levels of P-PRL in the milk during lactation (25, 26). Analysis of the involvement of different signaling pathways initiated by each of the two recombinant PRLs therefore may help to shed light on how PRL exerts different effects on the mammary gland in pregnancy versus lactation. Although, in this regard, it is clear that steroid hormones inhibit  $\beta$ -casein expression during pregnancy (27, 28), previous work from this laboratory has shown that the form of PRL also plays a role. This differential effect of the two PRLs is not via an effect on estrogen, progesterone, or corticosterone (1).

Substitution of a normally phosphorylated serine by an aspartate produces a residue similar in charge and size to a phosphorylated serine; the mimic of the phosphorylated molecule avoids the disadvantage of potential dephosphorylation. This approach is used widely in the study of enzymes

turned constitutively on or off by phosphorylation (e.g., ref 14). Here, we have used the molecular mimic of P-PRL, S179D PRL, to prevent possible interconversion of P-PRL and U-PRL during experimental procedures. Only with this approach can we definitively study the true differences in signaling between the two PRLs.

With U-PRL and the standard human PRL, one can observe signaling via Jak 2 and Stat 5 tyrosine phosphorylation as has now been described by many authors (reviewed in ref 10). Dimers of tyrosine-phosphorylated Stat 5 are capable of entering the nucleus and binding to GAS sites on the  $\beta$ -casein gene promoter (reviewed in ref 29). Evidence of similar signaling was present upon stimulation of the cells with S179D PRL but to a reduced degree. When an oligonucleotide equivalent to the  $\beta$ -casein GAS site was used for EMSA analysis, however, complex formation appeared qualitatively and quantitatively similar, regardless of the PRL used. Competition with antibodies showed that the majority of the complex formed in cells stimulated with the PRLs contained Stat 5a.

There is therefore an apparent contradiction in our results: The form of PRL which results in the highest level of  $\beta$ -casein mRNA, S179D PRL, is the one that least efficiently causes tyrosine phosphorylation of Stat 5a, and yet equivalent amounts of Stat 5a complexes are formed with the  $\beta$ -casein GAS site. Phosphoamino acid analysis of equivalent amounts of Stat 5 after stimulation with each PRL showed a doubling to tripling of phosphoserine content with S179D PRL. This suggests that serine phosphorylation of Stat 5 promotes Stat 5– $\beta$ -casein promoter complex formation and/or stability. Serine phosphorylation of other Stats has been shown to produce more stable Stat–DNA complexes and hence to improve transcriptional efficiency (e.g., ref 30), but some others have not found serine phosphorylation of Stat 5 in response to PRL (16). When comparing the current result with these others in the literature, however, it is important to note that U-PRL had no apparent effect on Stat 5 serine phosphorylation. Most other investigators use either recombinant PRL or various preparations of standard pituitary PRL from a variety of species. The proportion of pituitary PRL that is phosphorylated will vary from preparation to preparation, but the majority of what is present will always be U-PRL. The increase in serine phosphorylation with S179D PRL, which would be equivalent to 100% P-PRL, is large enough that it can be clearly observed over the inherent errors in this kind of experiment. It is entirely reasonable therefore that others have failed to find an effect of PRL on Stat 5 serine phosphorylation (16). In agreement with the current results showing PRL stimulation of Stat 5 serine phosphorylation are those of Yamashita et al., which show human PRL-stimulated serine phosphorylation of Stat 5 in both COS-7 and Nb2 cells and that this was accomplished using a proline-juxtaposed serine kinase (31). Thus S179D PRL, as a molecular mimic of P-PRL, results in stimulation of both tyrosine and serine phosphorylation of Stat 5, a maneuver which may be responsible for similar  $\beta$ -casein GAS site binding when compared to the more highly tyrosine phosphorylated Stat 5 produced by U-PRL. Is this sufficient, however, to result in superior  $\beta$ -casein gene expression? Work from the Waxman laboratory would suggest, if anything, that serine phosphorylation of Stat 5a is inhibitory to  $\beta$ -casein expression since mutation of serines

to alanines in Stat 5 enhanced expression of an intact  $\beta$ -casein promoter–luciferase reporter (32) in response to PRL. This is consistent with the idea that phosphorylation of the serines in Stat 5 is inhibitory to transcription. This same result was found in a second study by Yamashita et al. (33). On this latter occasion, however, the investigators additionally determined that the inhibition was removed by incubation in glucocorticoids, and all of our experiments were conducted in cells preincubated in, and in the continual presence of, glucocorticoids.

Evidence in the current paper supports some substrates of MAP kinase as key regulators of increased  $\beta$ -casein gene expression in response to S179D PRL. Thus S179D PRL, which promotes superior  $\beta$ -casein gene expression (versus U-PRL), is the better activator of MAP kinase. Further, incubation of cells with PD98059 blocked S179D PRL-stimulated  $\beta$ -casein expression. Activation of the MAP kinase pathway can result in activation of CREB and ATF<sub>1</sub>, both of which have consensus sequences/binding sites within the  $\beta$ -casein promoter (29). In addition, superior activation of  $\beta$ -casein gene expression can be achieved by removal of suppression by the transcription factor YY1 (29). Increased  $\beta$ -casein gene expression may therefore be elicited by interactions among all of these transcription factors. Once again, it is important to note that inhibition of MAP kinase had no effect on U-PRL-stimulated  $\beta$ -casein gene expression and hence that our results are not in conflict with previous reports that MAP kinase signaling has no role in PRL activation of  $\beta$ -casein gene expression (16). Instead, it is mostly the advantage afforded us by the use of S179D PRL that allows us to see this effect. When comparing to some literature reports, however, it is also important to note the use of a reporter construct assay system by some other investigators, even when using HC11 cells. The usual construct contains only the –344/–1 portion of the  $\beta$ -casein promoter (e.g., ref 16), and there are CREB, ATF<sub>1</sub>, and YY1 sites outside of this region. By contrast, we have used the endogenous gene for our analysis. In addition, it is also important to note that we have looked at steady-state mRNA levels after a long incubation in hormone. Effects on mRNA stabilization therefore may also be of consequence since several investigators have reported effects of various agents, including PRL, on the stabilization of  $\beta$ -casein transcripts (34–36). Ongoing work in the laboratory is designed to determine whether S179D PRL affects mRNA stability or transcription or both.

Long-term incubation in S179D PRL upregulated MAP kinase signaling in the cells and upregulated  $\beta$ -casein gene expression. At the same time, long-term incubation in S179D PRL also upregulated the short PRL receptor. This result suggests, but does not prove, that signaling from the short receptor is responsible for the increased MAP kinase activation seen with S179D PRL. Signaling from the short receptor, however, cannot be responsible for reduced activation of Stat 5a since long-term incubation in U-PRL also upregulated the short receptor to some degree while concurrently upregulating signaling through tyrosine phosphorylation of Stat 5a. Thus, we must conclude that the effect of S179D PRL on Stat 5a activation is a consequence of altered signaling at the long receptor: altered signaling due to the different conformation of the receptor after binding S179D PRL. This dual effect on the long and short receptor may be

the mechanism whereby S179D PRL can both inhibit growth in the mammary gland and also stimulate  $\beta$ -casein gene expression (1). Since we know that the short PRL receptor alone cannot activate  $\beta$ -casein gene expression (6), it is clear that a certain degree of Stat 5a tyrosine phosphorylation must be crucial. Apparently, this does not have to be very large since the activation shown after a 7-day incubation in S179D PRL is much weaker than that seen with U-PRL. Presumably, this reflects a large excess of Stat 5a phosphorylation by U-PRL and reminds us that signaling experiments usually use supraphysiological concentrations of ligand in order to see a robust effect. Titration of S179D PRL against a fixed dose of U-PRL demonstrated competition for tyrosine phosphorylation of Stat 5a. Thus, when used alone, S179D PRL is a partial agonist for Stat 5a tyrosine phosphorylation. However, when used together with the more potent tyrosine phosphorylator of Stat 5a, U-PRL, it acts as an antagonist. At physiological ratios of U-PRL to P-PRL (equivalent to lanes 2 and 3 on Figure 6), there is clear modulation of Stat 5 tyrosine phosphorylation.

The results presented here do not agree with previous reports by other investigators that the short receptor acts as a dominant negative for signaling to  $\beta$ -casein gene expression (37). The dominant negative effect was obtained in multiply transfected overexpression systems using the single long and short forms of the rat PRL receptor. Using essentially the same approach, we have duplicated the dominant negative result in CHO cells but with the same reporter construct find S179D PRL to result in greater luciferase activity in HC11 cells (data not presented). There is therefore a significant difference between these systems and natural regulation of  $\beta$ -casein expression in HC11 cells. This may involve the presence of specific signaling molecules or molecules that act as transcription cofactors, an important possibility in light of the fact that HC11 cells are the only mammary cell line capable of expressing endogenous  $\beta$ -casein. Also, if expression of the short receptor exceeds physiological ratios with the long receptor or total receptor expression is very high (as could be produced in the overexpression systems), heterodimer formation between long and short receptors may well artifactually result in an inhibition of active long and short receptor dimers and therefore would inhibit  $\beta$ -casein gene expression. In keeping with our contention that upregulation of the short receptor is beneficial to milk protein gene expression is a previous report of a 2-fold upregulation of the short receptor between pregnancy and lactation in rats (4). In addition, recent work from the Kelly group has shown replacement of one of the three short forms of the mouse PRL receptor to rescue lactation in the PRL receptor knockout heterozygous animal, whereas the other forms acted as dominant negatives (38). Perhaps, therefore, transfection of the rat short form of the receptor into CHO cells cannot duplicate the appropriate short form in mouse HC11 cells. Moreover, in the ovary, it is clear that the short PRL receptor has a distinct function and that it does not act simply as a dominant negative form (39).

In conclusion, these results underline the limitations of transfection and reporter construct analyses. More importantly, they demonstrate that U-PRL and S179D PRL signal differently in HC11 cells and that the differences in their signaling can be amplified by long-term exposure. U-PRL primarily uses the Jak–Stat pathway, while S179D PRL

primarily uses the MAP kinase pathway, although both PRLs use both pathways to some extent. In addition, the results suggest a novel and important role for natural expression levels of the short PRL receptor in the enhancement of  $\beta$ -casein expression, a finding currently under further investigation.

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## **Pseudophosphorylated Prolactin Up-regulates p21 and Vitamin D Receptor.**

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Running Title: Phospho PRL upregulates p21/waf1 and vitamin D receptor

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## **Abstract**

Recent work has demonstrated that unmodified prolactin (U-PRL) stimulates mammary growth, while a molecular mimic of phosphorylated PRL (S179D PRL) inhibits growth. In this study, we have examined key cell cycle regulatory proteins previously implicated in the control of mammary cell proliferation. Treatment with U-PRL resulted in increased expression of cyclin D1 and the kinase it activates, cyclin-dependent kinase 4 (cdk4), while there was no effect on the cell cycle inhibitory protein, p21. S179D PRL, by contrast, had no effect on cyclin D1 and cdk4, while increasing expression of p21. S179D PRL also elevated expression of the vitamin D receptor (VDR), which is itself known to elevate p21. Conduct of the experiments in medium depleted of vitamin D suggests that liganded VDR is not required for the effect of S179D PRL on p21. Both up-regulation of p21 and VDR by S179D PRL were mediated through the MAP kinase pathway, thus suggesting that up-regulation of the VDR is an independent consequence of MAP kinase activation.

**Keywords:** prolactin, phosphorylated prolactin, cell cycle regulatory proteins, vitamin D, Vitamin D receptor, MAP kinase, cyclin D, cyclin dependent kinase 4, p21

## Introduction

Since the discovery of phosphorylated prolactin (P-PRL) in our laboratory [1], we have investigated the physiological regulation of the amount phosphorylated [2,3], and the different biological activities of unmodified prolactin (U-PRL) versus P-PRL [4,5]. In these studies, we determined that P-PRL acted as an antagonist to U-PRL-stimulated growth in two different cell lines [4,5], and that treatment of a pituitary tumor cell line with P-PRL caused the cells to take on a more differentiated phenotype [4]. More recently, we have investigated the biological activities of U-PRL and P-PRL in vivo, utilizing recombinant versions of each. In order to make recombinant P-PRL, we synthesized a molecular mimic in which the normally phosphorylated serine [6,7] was replaced with an aspartate residue, producing S179D PRL [8]. Like P-PRL, S179D PRL antagonizes U-PRL-stimulated growth [8-13]. At the same time, S179D PRL, like P-PRL [4], stimulated differentiated function [11,13]. In the mammary gland, S179D PRL inhibited U-PRL-promoted growth and yet also promoted the formation of alveoli and a high level of beta-casein expression within these alveoli [11]. Thus we have hypothesized that it is a balance between the proliferative [5] and antiapoptotic effects [10,14,15] of U-PRL and the anti-proliferative [4,5,8-12] and pro-apoptotic [10] and differentiative [4,11,13] effects of P-PRL that determines the response of mammary tissue to circulating total PRL. With higher proportions of U-PRL, growth predominates over differentiated function. With increasing proportions of P-PRL comes decreased growth and increased differentiated function. Too much U-PRL might result in unregulated growth, whereas too much P-PRL has been shown to result in insufficient growth and lactational failure [11,16].

As part of a study to determine whether an increased ratio of U-PRL: P-PRL would promote mammary tumorigenesis, we have examined the effect of each PRL on key cell cycle

regulating proteins. Moreover, we have examined this issue using a protocol designed by others to promote differentiative responses to PRL [17]. In this way we aim to more closely replicate initiation of abnormal growth in normal cells rather than promotion of growth of cells that are already tumorous.

Using preparations of PRL that contain both U-PRL and P-PRL, others have reported PRL stimulation of expression of cyclin D1 [18,19] and the vitamin D receptor (VDR) [20]. Vitamin D upregulates the VDR [21] and induces cell cycle arrest through upregulation of the cell cycle inhibitor, p21 [21-23]. We have therefore investigated the individual effects of U-PRL and P-PRL on cyclin D1 and the kinase it activates, on the VDR and on p21.

We demonstrate that the effects of U-PRL on the expression of cyclin D1 and cdk4 are consistent with promotion of cell cycle progression and that the effects of S179D PRL on the expression of p21 and the VDR are consistent with inhibition of cell cycle progression.

## MATERIALS AND METHODS

### Production of recombinant PRLs

Both recombinant human U-PRL and S179D PRL were produced and characterized as previously described [8]. The proteins were expressed, purified and tested for their activity in an Nb2 cell bioassay. U-PRL promotes Nb2 cell proliferation, whereas S179D PRL antagonizes this effect.

### HC11 cell culture

HC11 cells are a normal mouse mammary cell line, which under appropriate conditions will produce the major milk protein, beta-casein, in response to added PRL [17]. The HC11 cells were a gift from Nancy Hynes (Friedrich Miescher Institutes, Basel, Switzerland). HC11 cells were routinely grown in RPMI 1640 growth medium (Gibco BRL, NY) containing 10% FBS (75pg calcitriol/ml serum), 5 $\mu$ g/ml insulin (Sigma, St. Louis, MO), 10ng/ml epidermal growth factor (Gibco BRL), 100U/ml penicillin, and 100 $\mu$ g/ml streptomycin. Once confluent, they were grown for 3 more days in growth medium. The medium was changed daily. On the third day post-confluence, the growth medium was removed, and the cells were washed 5 times with RPMI 1640 basal medium. The cells were refed with priming medium. Priming medium was RPMI 1640 basal medium supplemented with 10% charcoal stripped horse serum (approximately 40pg/ml serum calcitriol) (Cocalico Biologicals, Reamstown, PA), 100 U/ml penicillin, 100 $\mu$ g/ml streptomycin, 10 $\mu$ g/ml insulin, and 1 $\mu$ g/ml hydrocortisone (Sigma). The cells were kept in priming medium for 24 h. The cells were then refed with experimental medium. The experimental medium was the priming medium to which 1 $\mu$ g/ml of the appropriate

PRL was added. This protocol essentially follows Taverna et al. and is designed to produce a contact-inhibited monolayer of relatively differentiated cells [17]. In the present studies, cells were maintained in experimental medium for 3 or 7 days. These time points were chosen for two reasons. First, others have shown that it takes 5-7 days of treatment with PRL to see optimal differentiative responses in these cells [24]. Second, we aimed to investigate long-term, sustained responses which might contribute to sustained effects relevant to tumor initiation. Cells were then collected for protein extraction. In the MAP kinase experiments, the MAP kinase inhibitor PD98059 (25 $\mu$ M) was included. This concentration of PD98059 has been shown to efficiently inhibit MAP kinase activity in HC11 cells [25].

#### **Preparation of whole cell, cytosolic and nuclear extracts**

Cells were rinsed with phosphate buffered saline (PBS) and scraped off the plate in a buffer containing 20mM Tris-HCl, pH7.4, 140mM NaCl, 0.05mM EDTA, 10 $\mu$ g/ml Leupeptin, 10 $\mu$ g/ml Aprotinin, 25 $\mu$ g/ml Pepstatin, 1mM PMSF, 1mM Na<sub>3</sub>VO<sub>4</sub>, 10nM NaF, 1mM EGTA and 1% NP-40. The cell lysate was homogenized and centrifuged at 12,000g for 5 min, and the supernatant was considered a whole cell extract. If the cells were scraped into hypotonic buffer (10mM Tris pH 7.4, 10mM NaCl, 6mM MgCl<sub>2</sub>, 1mM dithiothreitol, and 0.1 mM Na<sub>3</sub>VO<sub>4</sub>) and disrupted with a Dounce homogenizer, the supernatant after centrifugation at 12,000g for 5 min was considered a cytosolic extract. When the pellet from this centrifugation was resuspended in 3 volumes of hypertonic extraction buffer (20% glycerol, 20mM Hepes, pH 7.9, 420mM NaCl, 1.5 mM MgCl<sub>2</sub>, 0.2 mM EDTA, 0.2 mM phenylmethylsufonyl fluoride, 1mM dithiothreitol, and 0.1 mM Na<sub>3</sub>VO<sub>4</sub>) and incubated on ice for 30min, the supernatant obtained after

centrifugation was considered a nuclear extract. Protein concentration was measured by the Bradford method.

### Western blot

Ten or 20 $\mu$ g protein were loaded onto a reducing SDS-PAGE gel. After electrophoresis, protein was transferred to nitrocellulose membranes in 48mM Tris, 39mM glycine, 0.1% SDS and 20% methanol (pH 8.3). Membranes were blocked with 5 % nonfat milk in wash buffer consisting of Dulbecco's phosphate-buffered saline (Gibco BRL) and 0.1% Tween 20. Blotted and blocked membranes were probed with primary rabbit polyclonal Ig Gs for cyclin-dependent kinase 4 (cdk4) (1: 1000), p21 (1:500) or VDR (1:2000) (Santa Cruz Biotechnology, CA) or mouse monoclonal IgG1 for cyclin D1 (1: 500) in wash buffer for 3 h at room temperature or overnight at 4C. After washing 3 times for 15 min each, the blots were incubated in goat anti-rabbit or anti-mouse IgG conjugated to horseradish peroxidase (Sigma) at a dilution of 1: 2000-1:10000, as appropriate, for 30-45 min at room temperature. After 3 washes, membranes were treated with ECL reagent (Amersham Biosciences) followed by autoradiography. After detection of cytosolic proteins, blots were stripped in wash buffer with  $\beta$ -mercaptoethanol (7 $\mu$ l/ml) and 2% SDS for 30min at room temperature with agitation, followed by washing for 30min. After reblocking, the blots of whole cell or cytosolic extracts were reprobed with mouse monoclonal IgG for  $\beta$ -actin (1:5000) (Sigma) to normalize for loading and transfer. All blots included molecular weight markers and were exposed multiple times to ensure film development in the linear range. Controls included the use of non-specific antibodies in place of the primary antibody.

### **Statistical analysis**

Data were subjected to analysis of variance with posttests for comparison among specific groups. Bonferroni corrections for multiple comparisons against a single group were used. The minimum number of repetitive experiments was 3.

## RESULTS

### Effect of each PRL on confluent, primed cells

Work from other laboratories has produced a protocol which causes cell differentiation and sensitizes HC11 cells to PRL promotion of  $\beta$ -casein gene expression [17]. We have used this protocol previously to demonstrate that S179D PRL is more effective at increasing steady-state  $\beta$ -casein mRNA levels than U-PRL [11,26]. Here we are examining the effect of each PRL on cell cycle regulatory proteins in these relatively non-proliferative cells. Since we had previously used a 7-day incubation when examining  $\beta$ -casein expression in response to each PRL and this is indicative of a differentiative response, we began our studies with this incubation time. In this time frame, S179D PRL increased the amount of the cyclin-dependent kinase inhibitor, p21 in the cell nuclei (figure 1). Repetition of this part of the experiment utilizing a 3-day incubation is shown in figure 2, where S179D PRL caused a 75% increase in the amount of nuclear p21. A 7-day incubation in U-PRL by contrast, had no effect on the amount of nuclear p21 (figure 1), but increased the amount of nuclear cyclin D1 by 50% (figure 3). Also elevated by U-PRL was cdk4 (figure 4), a 90% increase. At the same time, S179D PRL was without effect on the levels of cyclin D1 and cdk4 (figures 3 & 4). Thus, U-PRL elevated nuclear levels of the cell-cycle-promoting cyclin D1 and the kinase it activates, while S179D PRL increased levels of the cell cycle inhibitor which blocks the downstream effect of the cyclin D1-cdk4 complex.

Vitamin D inhibits mammary cell proliferation [27-29] and promotes cell differentiation [21,22] and these effects are mediated through p21 [30], we therefore examined the possibility that the downstream effects of S179D PRL were mediated through the VDR. A 3-day incubation in S179D PRL increased VDR expression by 50-60% (figures 5 and 6) and increased VDR

nuclear translocation (figure 7). In fact all of the additional VDR protein was localized to the nucleus (figure 7). Of significance, however, is the fact that these experiments were conducted using charcoal-stripped serum with an approximate incubation concentration of 2 pg/ml 1,25 dihydroxyvitamin D3.

A previous study had demonstrated that S179D PRL signaled to produce increased  $\beta$ -casein mRNA levels primarily through the MAP kinase pathway [26]. We therefore also examined whether the MAP kinase pathway was involved in the elevation of nuclear p21 and VDR in response to S179D PRL. Figure 2 demonstrates that the 75% increase in nuclear p21 in response to S179D PRL can be blocked by the MAP kinase inhibitor, PD98059, whereas there was no effect of this compound on the level of nuclear p21 in the controls. Likewise, PD98059 blocked the 60% increase in expression of the VDR (figure 6) in response to S179D PRL.

## DISCUSSION

Previous work from our laboratory has demonstrated that S179D PRL inhibits growth and promotes the expression of tissue-specific genes [8-13]. Thus S179D PRL inhibits the growth of human prostate cancer cells both *in vivo* and *in vitro* [12] and promotes the expression of prostatein and probasin in the normal rat prostate [13]. Likewise, S179D PRL inhibits pregnancy-induced growth of the mammary gland and promotes the expression of  $\beta$ -casein [11]. On the other hand, U-PRL has been demonstrated to promote growth of human prostate cancer cells [12] and to reduce expression of prostatein and probasin in the normal rat prostate [13]. In the pregnant mammary gland, U-PRL stimulates growth and reduces the expression of  $\beta$ -casein [11].

By using a cell line, which can be caused to slow its growth and to become capable of expressing  $\beta$ -casein, we can examine the effect of these two PRLs in a homogenous *in vitro* system somewhat akin to normal tissue. The ability to express  $\beta$ -casein in response to PRL is brought about by initial growth in the presence of EGF and insulin to postconfluence and then treatment with hydrocortisone in the continued presence of insulin [17]. Under these conditions, it takes 5-7 days of PRL treatment to see optimal changes in the amount of  $\beta$ -casein expressed [24]. Similar conditions were therefore used to examine effects on cell cycle regulatory proteins since our aim was to determine sustained effects on relatively differentiated cells.

We found S179D PRL to increase nuclear p21, while having no effect on the levels of cyclin D1 or the kinase it activates, cdk4. Since cdk4-cyclin D1 complexes, through phosphorylation of restriction period (Rb) proteins, cause the synthesis of cyclin E [reviewed in 31] and p21 inactivates cdk2-cyclin E complexes [reviewed in 32], this result suggests that S179D PRL exerts its effect on the cell cycle at a post restriction period stage. U-PRL, by

contrast, has no effect on nuclear p21, while elevating cyclin D1 and cdk4. This suggests an earlier and distinct effect on the cell cycle regulatory mechanism.

Other investigators have demonstrated that PRL increases cyclin D1 levels primarily through the Jak2-Stat5 signaling pathway [19]. This is in accord with the current results and with a previous publication from this laboratory which shows that U-PRL primarily activates the Jak2-Stat5 pathway in HC11 cells [26]. By contrast, this same previous publication showed S179D PRL to signal primarily through the MAP kinase pathway [26]. Numerous studies have demonstrated that sustained MAP kinase activation results in p21 upregulation [33-36]. Overexpression of p21 can inhibit mammary carcinoma growth both *in vivo* and *in vitro* [37]. Here we demonstrate that blockade of the MAP kinase pathway, blocks the increase in nuclear p21 in response to S179D PRL, while having no effect on the levels of nuclear p21 in control cells. Combining our present results with those of others [19] once again suggests differential primary signaling pathways for these two forms of PRL, the Jak2-Stat5 pathway being used by U-PRL to promote progression through the cell cycle and the MAP kinase pathway being used by S179D PRL to interfere with cell cycle progression by inactivating cdk2-cyclin E complexes.

Vitamin D has received a lot of attention in recent years because of its ability to inhibit mammary cell growth and to promote differentiation and apoptosis [21-23,27-30]. Because of the apparent similarities in the end effects of vitamin D and S179D PRL, as well as the presence of a VDR binding site in the p21 promoter, it was of interest to examine the effect of S179D PRL on mammary expression of the VDR. S179D PRL, but not U-PRL, increased expression of the VDR and resulted in increased nuclear translocation of the VDR. Like the effect on p21, this was inhibited by the MAP kinase inhibitor, PD 98059. Thus elevation of both p21 and VDR expression involve use of the MAP kinase signaling pathway. It is possible therefore that some

effects of S179D PRL are mediated through the VDR, although this remains to be demonstrated. It is unlikely, however, that the effect of S179D PRL on p21 expression occurs only via making the cells more sensitive to vitamin D since the culture medium used for the current experiments contained only about 2pg/ml of this vitamin/hormone and anti-proliferative responses in cell lines are usually observed in the ng/ml range [27,29]. It is therefore unlikely that the effect of S179D PRL on p21 occurs via a liganded VDR. This raises the possibility that dual treatment with S179D PRL and vitamin D might be more efficacious at controlling mammary cell proliferation than either compound alone. If there is insufficient vitamin D in the medium, what therefore causes nuclear translocation of the VDR? Others have demonstrated nuclear shuttling of unliganded VDR [38] and unliganded VDR can exert effects via heterodimerization with the retinoid X receptor (RXR) [39], so the possibility still exists that the effects of S179D PRL are mediated through an unliganded VDR. More likely in our opinion, however, are overlapping effects of S179D PRL and vitamin D at the level of MAP kinase activation; S179D PRL activates MAP kinase and vitamin D activates MAP kinase [40]. In support of this concept are publications showing the differentiative effects of vitamin D to be mediated through the MAP kinase pathway in leukemia cells [40]. In addition, p21 induction occurs through the MAP kinase pathway [33-35].

S179D PRL was used in the current study both to examine the physiological role of phosphorylated PRL with a tool that cannot be dephosphorylated during the experimental protocol and to gain insight into the mechanism of action of S179D PRL, a molecule which may prove to be a potential therapeutic for abnormal mammary cell proliferation. In the former role, the current work suggests that the balance between U-PRL and P-PRL physiologically is important in regulating cell cycle progression and is consistent with previous studies which

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Fig. 1

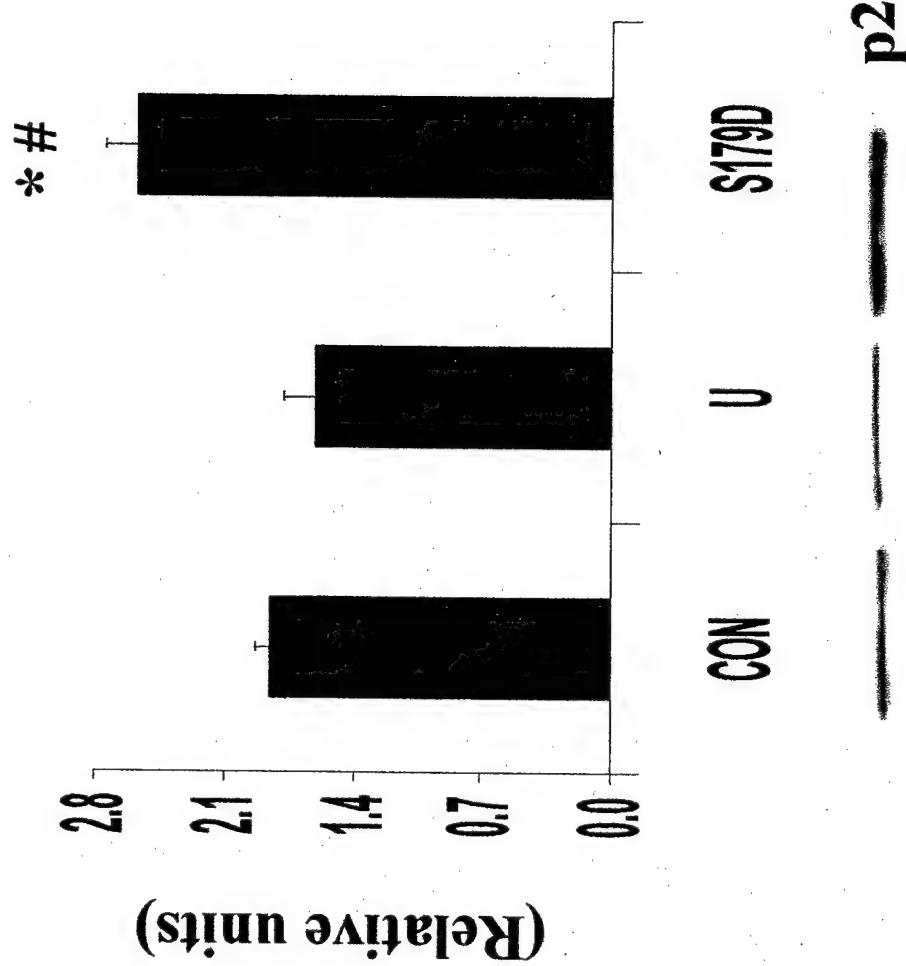


Fig. 2

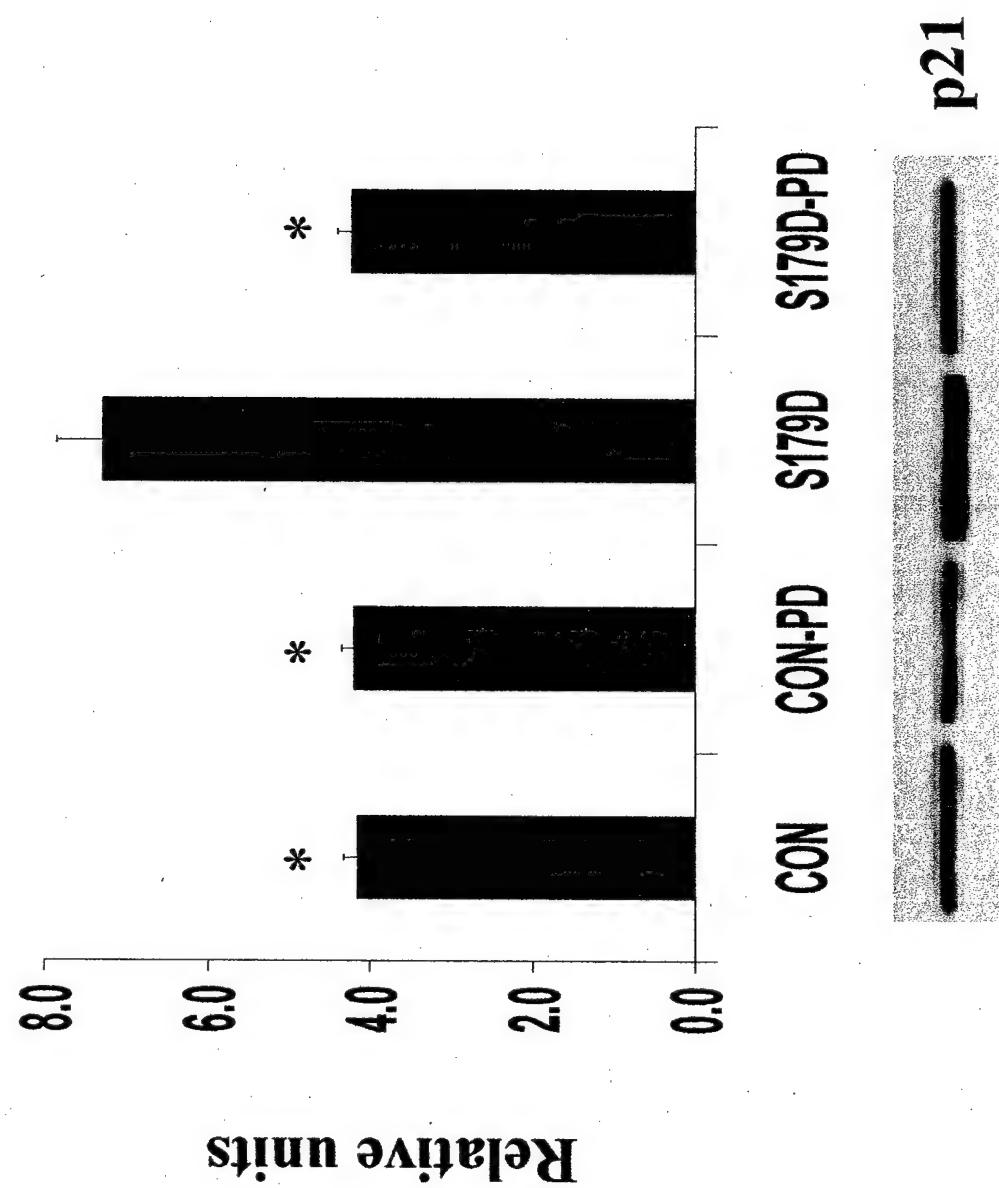


Fig.3

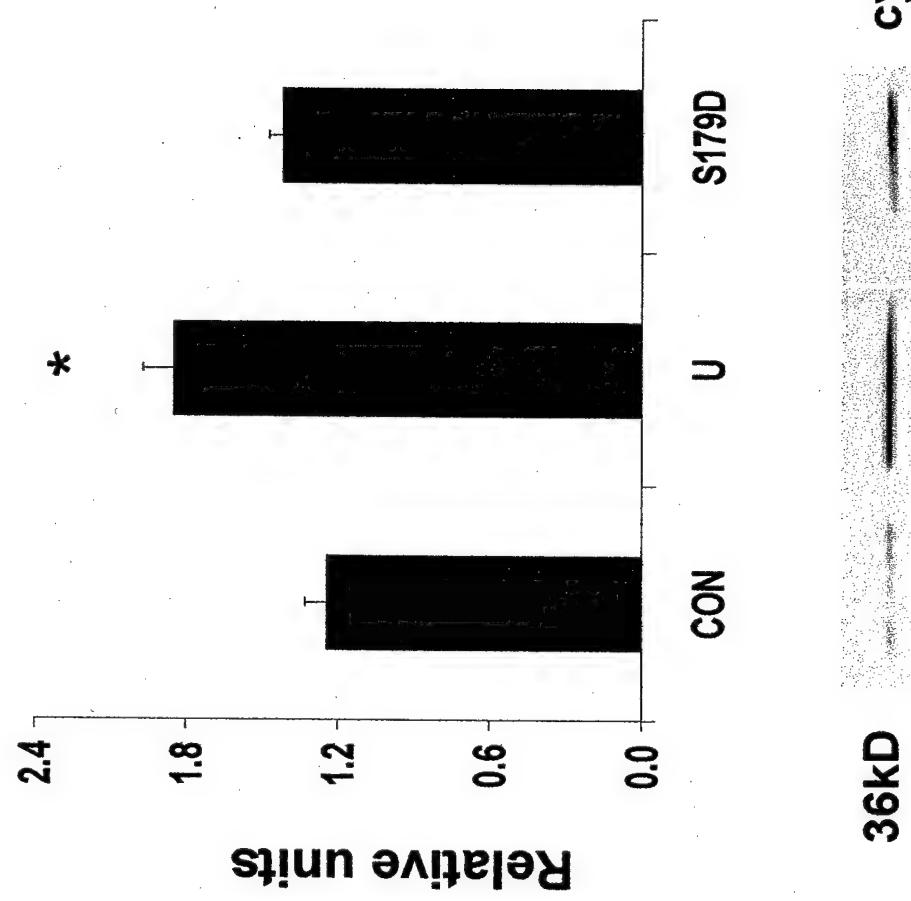
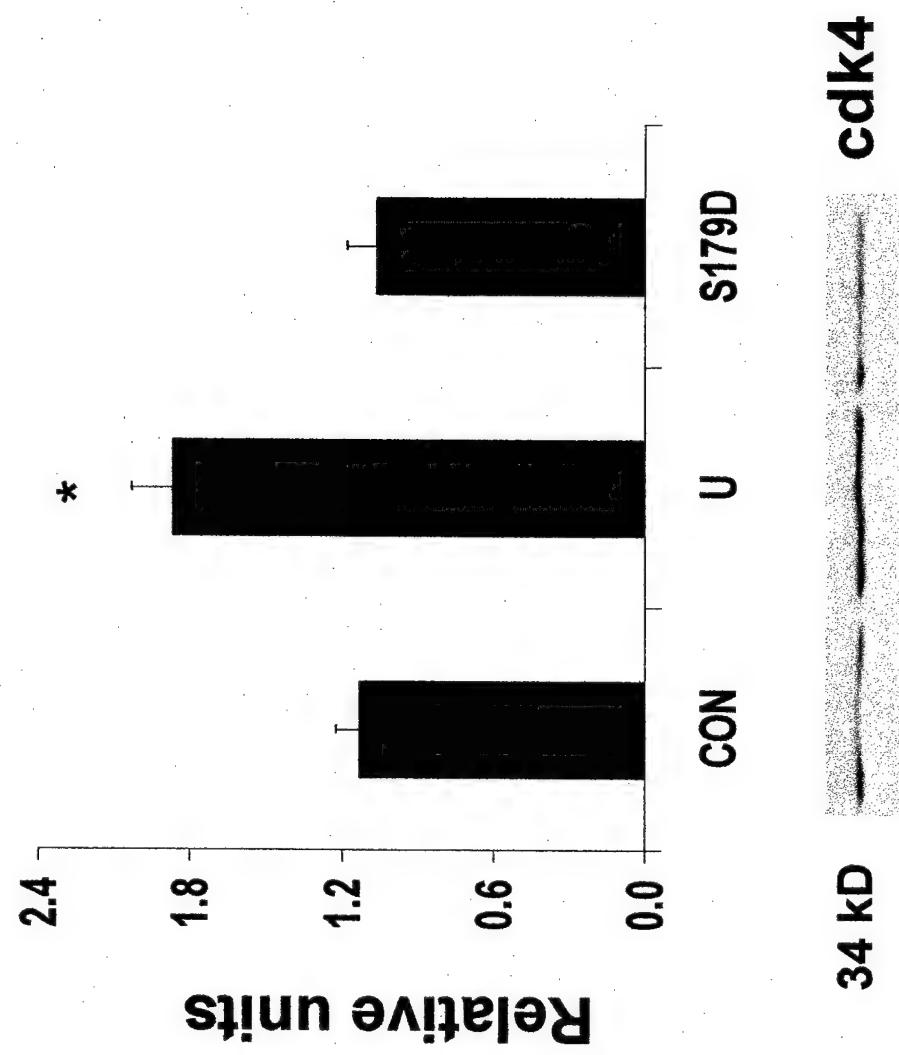


Fig. 4



**Fig. 5**

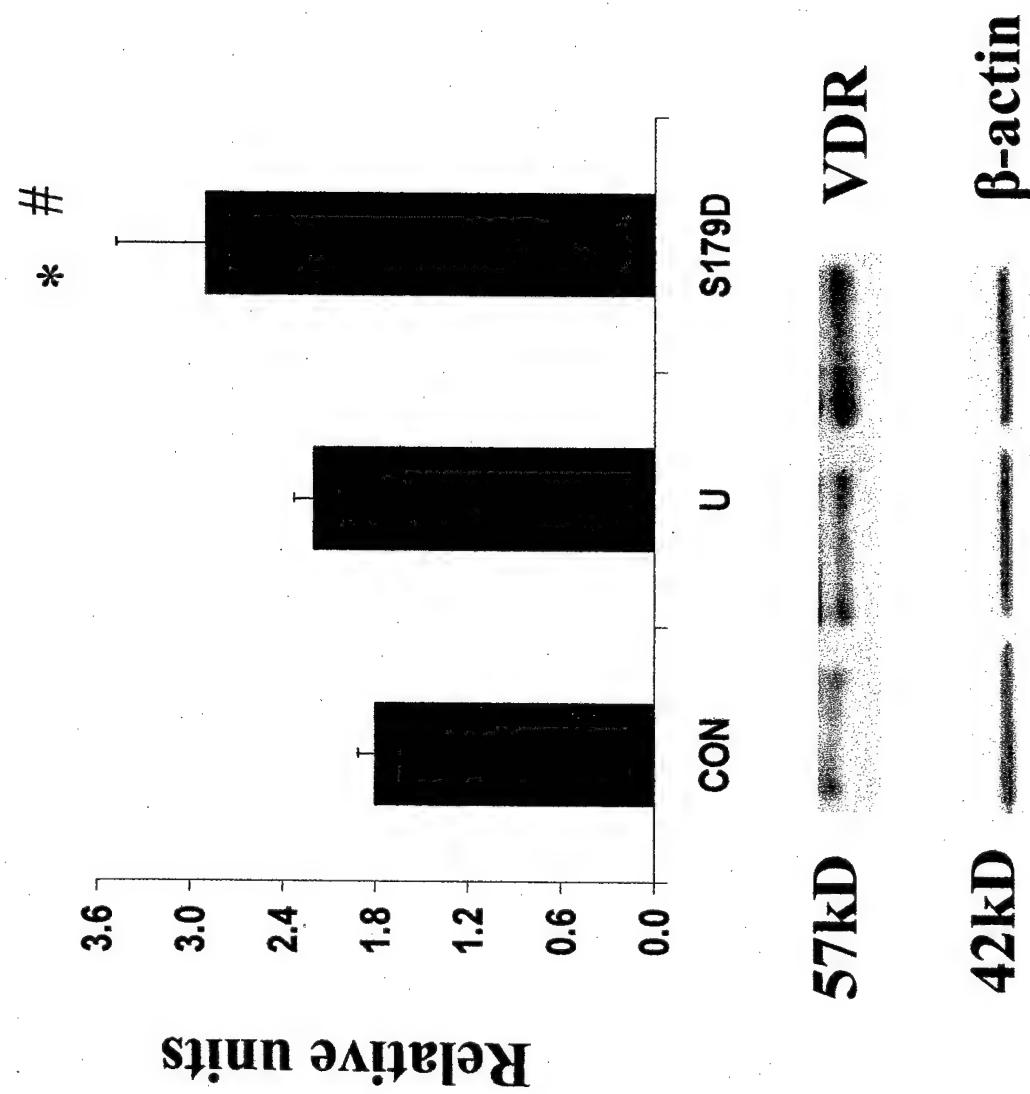
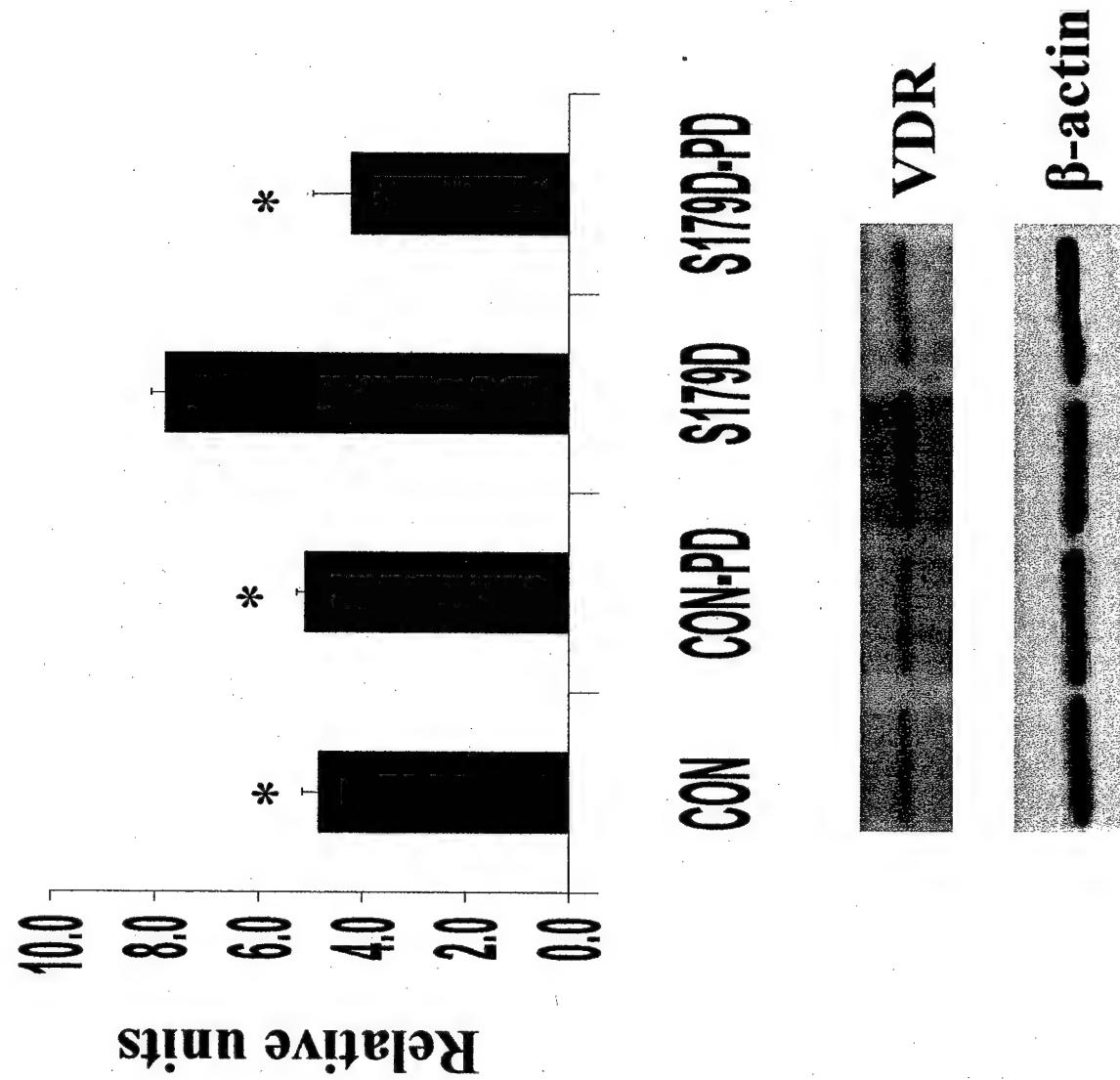
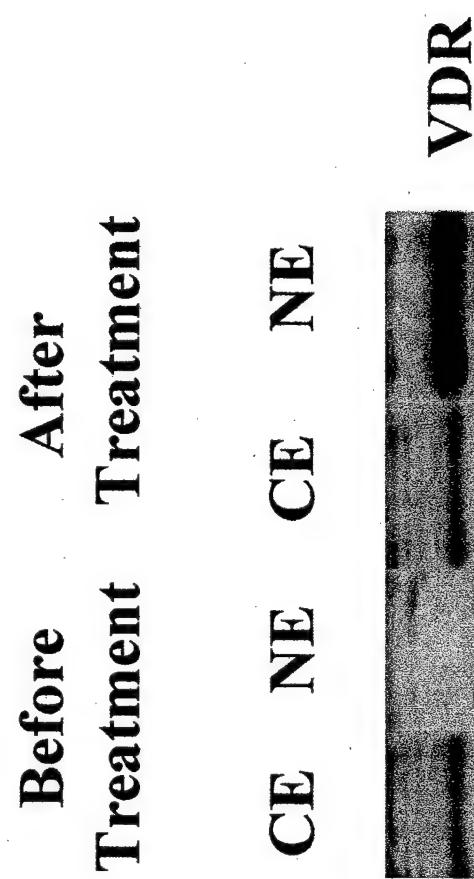


Fig. 6



**Fig.7**



## FIGURE LEGENDS

**Figure 1.** Up-regulation of p21 in S179D PRL-treated HC11 cells. Cells were treated with each PRL for 7 days. Ten  $\mu$ g of HC11 cell nuclear extracts were analyzed in the different treatment groups. CON, control; U, U-PRL; S179D, S179D PRL. Rabbit polyclonal anti-p21 was used for Western blotting. \*, significantly different from control cells ( $p<0.05$ ); #, significantly different from U-PRL treated cells ( $p<0.01$ ). Values are mean  $\pm$  SE (n=6).

**Figure 2.** Effect of MAP kinase inhibition on S179D PRL-induction of p21. HC11 cells were treated with S179D PRL for 3 days in the absence and presence of the MAP kinase inhibitor, PD98059. Note that PD98059 inhibited p21 up-regulation induced by S179D PRL; CON, control cells; CON-PD, control cells treated with PD98059; S179D, S179D PRL treated cells; S179D-PD, cells treated with both S179D PRL and PD 98059. \*, significantly different from S179D PRL treated cells ( $p<0.01$ ). Values are mean  $\pm$  SE (n=6).

**Figure 3.** Up-regulation of cyclin D1 in U-PRL-treated HC11 cells. Cells were treated with each PRL for 7 days. Ten  $\mu$ g of HC11 cell nuclear extract were analyzed in the different treatment groups. CON, control; U, U-PRL; S179D, S179D PRL; 36kD, estimated molecular mass of cyclin D1 based on co-run reference markers. Mouse

monoclonal anti-cyclin D1 was used for Western blotting. \*, significantly different from control cells ( $p<0.05$ ). Values are mean  $\pm$  SE ( $n=6$ ).

**Figure 4.** Up-regulation of cdk4 in U-PRL-treated HC11 cells. Cells were treated with each PRL for 7 days. Twenty  $\mu$ g of HC11 nuclear extract were analyzed in the different treatment groups. CON, control; U, U-PRL; S179D, S179D PRL; 34 kD, estimated molecular mass of cdk4 based on co-run molecular markers. Rabbit polyclonal anti-cdk4 was used for Western blotting. \*, significantly different from control cells ( $p<0.05$ ). Values are mean  $\pm$  SE ( $n=6$ ).

**Figure 5.** Up-regulation of VDR by S179D PRL in HC11 cells. HC11 cells were treated for 4 days with each PRL (same result at 5 and 6 days). Twenty  $\mu$ g of whole cell extracts were probed by Western blot using a rabbit polyclonal anti-VDR. Anti-  $\beta$ -actin was used to control for equal loading and transfer. \*, significantly different from control cells ( $p<0.01$ ); #, significantly different from S179D PRL treated cells ( $p<0.05$ ). Values are mean  $\pm$  SE ( $n=6$ ).

**Figure 6.** Effect of MAP kinase inhibition on S179D PRL-induction of VDR. Cells were treated with S179D PRL for 3 days in the absence and presence of the MAP kinase inhibitor, PD98059. Whole cell extracts were subjected to Western blotting as for figure 5. CON, control cells; CON-PD, control cells treated with PD98059; S179D, S179D PRL treated cells; S179D-PD, cells treated with both S179D PRL and PD 98059. \*,

significantly different from S179D PRL treated cells ( $p<0.01$ ). Values are mean  $\pm$  SE (n=6).

**Figure 7. Subcellular localization of the VDR following treatment with S179D PRL.**

Cells were treated with S179D PRL for 3 days. VDR was analyzed in both cytosolic and nuclear extracts before and after treatment with S179D PRL. Ten  $\mu$ g of cytosolic or nuclear extract were analyzed by Western blot. Results showed that S179D PRL increased the total amount of VDR, duplicating results shown in figures 5 & 6 and further that S179D PRL promotes VDR nuclear translocation. CE, cytosolic extracts; NE, nuclear extract.

**Inhibition of PRL-Induced Proliferative Signals in Breast Cancer Cells by a  
Molecular Mimic of Phosphorylated PRL, S179D-PRL**

Inhibition of PRL induced proliferation by S179D-PRL

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Key words-Prolactin, prolactin receptor, breast cancer, STAT 5, cyclin D1 and S179D-PRL

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Abbreviations: PRL, prolactin; lPRLR, long form of the prolactin receptor; iPRLR, intermediate form of the prolactin receptor

## ABSTRACT

Posttranslational modifications of prolactin (PRL), including phosphorylation, vary with physiologic state and alter biologic activity. In light of the growing evidence for a role for PRL in proliferation in mammary cancer, we examined the ability of a mimic of phosphorylated human prolactin, S179D-PRL, to initiate signals to several pathways in mammary tumor cells alone and in combination with unmodified PRL. In PRL-deficient MCF-7 cells, S179D-PRL was a weak agonist compared to unmodified PRL with regard to cellular proliferation, cyclin D1 levels, and phosphorylation of STAT 5 and ERKs. However, S179D-PRL was a potent antagonist of unmodified PRL to these endpoints. In contrast to the reduced levels of the long isoform of the PRLR observed in response to a 3d incubation with unmodified PRL, S179D-PRL upregulated expression of this isoform, 4 fold. These studies support the utility of this mutant as a PRL antagonist to proliferative signals in mammary epithelial cells, including a potential role in breast cancer therapeutics.

Although prolactin (PRL) exerts effects on many diverse physiological processes, one of the best studied targets is the mammary gland (for reviews, 1,2). PRL is critical for both mammogenesis and lactation, and accumulating evidence indicates that it may play a role in mammary carcinogenesis as well (for review, 3-5). In rodent models, systemic PRL treatment or pituitary transplants increased development of spontaneous, chemically induced, and oncogene-initiated mammary tumors (6-8), and mammary transgenic PRL induced tumors in virgin females (9,10). While the mechanism(s) whereby this occurs are not well understood, PRL increased bromodeoxyuridine incorporation in morphologically normal structures as well as epithelial hyperplasias and adenocarcinomas in the NRL-PRL transgenic and pituitary isograft models (10,11), suggesting that augmented proliferation contributes to the disease process. The role of PRL in human breast cancer has been controversial, since correlations between breast cancer development and progression, and circulating levels of PRL have been conflicting, and inhibition of pituitary PRL synthesis with bromocriptine did not alter the disease course (for review, 3). A more recent large prospective study by Hankinson et al., however, shows a correlation between PRL levels in the high normal range and increased risk of breast cancer (12). Furthermore, production of PRL within primate mammary epithelial cells themselves indicates that the pituitary may not be the only relevant source of this hormone (for review, 3,4). The high levels of PRL receptors (PRLR) in a large portion of tumors (13-15) suggest that the PRL signaling pathway may be a useful therapeutic target in human disease.

PRL can be modified post-translationally by various processes including phosphorylation and glycosylation, as well as proteolytic cleavage (for review, 16,17). The variability of these modifications with physiologic context prompted the hypothesis that they may contribute to the diverse effects of PRL at its targets, including the mammary gland (18,19). Phosphorylation, in particular, has been shown to decrease its mitogenic action in the PRL-dependent rat lymphoma cell line, Nb2 (18,20). In order to more easily study the activity of phosphorylated PRL, Walker and colleagues designed a phosphomimic, substituting an aspartate for serine at amino acid 179 of hPRL (S179D-PRL), a major site of phosphorylation (21,22). In their hands, this mutant was an effective antagonist for unmodified PRL-induced proliferation of Nb2 cells (22,23). However, Goffin and coworkers observed no evidence for antagonism and only weak agonistic activity using their own preparations of this mutant hormone in the same system (24). Recent studies analyzing development of the mammary gland during pregnancy and responses of mammary epithelial cells *in vitro* have demonstrated a complex spectrum of activity for S179D-PRL. S179D-PRL treatment during pregnancy inhibited murine alveolar growth (25,26), while concomitantly increasing  $\beta$ -casein transcripts (25). In the normal murine mammary cell line, HC11, which differentiates in response to PRL, Walker and colleagues found that S179D-PRL increased transcripts for  $\beta$ -casein relative to unmodified PRL, and that this was blocked by selective inhibitors of ERKs (27,28).

The effect of PRL on mammary cells that have already undergone neoplastic changes may be distinct from normal cells. Study of PRL actions in human mammary tumor cells has been hampered by the endogenous PRL production, which makes experimental

manipulation of PRL availability difficult. In order to provide a model to examine PRL signaling pathways and target genes in human tumor cells, we have derived PRL-deficient MCF-7 cells that demonstrate increased sensitivity to exogenous hormone (29). PRL increases proliferation of these cells by facilitating the G1/S transition, in part by increasing cyclin D1 expression and activity and decreasing p21 levels (29,30). Using this system and homologous hormone, here we have examined the signals transmitted by S179D-hPRL to several well characterized pathways, and its effect on the actions of unmodified PRL. Our data demonstrate that S179D-PRL is a weak agonist that is able to antagonize proliferative signals initiated by unmodified PRL in these cells.

## **Materials and Methods**

### *Materials*

Antibodies used in Western analyses were as follows: cyclin D1 (MS-210-P1) from NeoMarkers (Fremont, CA); STAT 5 pTyr<sup>694</sup> (71-6900), PRLR long form (35-9200) and PRLR intermediate form (34-4800) from Zymed Labs, Inc. (San Francisco, CA); STAT 5 (sc-835) from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), and phospho-p44/42 ERK (Thr202/Tyr204) (9101) and p44/42 ERK (9102) from NEB Cell Signaling (Beverly, MA). The enhanced chemiluminescence kit was purchased from Amersham Pharmacia Biotech (Arlington Heights, IL). All of the remaining reagents were purchased from Sigma-Aldrich Corp. (St. Louis, MO). Recombinant WT-PRL and S179D-PRL were prepared as previously described (22,24). Data presented are from experiments performed with S179D-PRL synthesized in the Walker laboratory unless otherwise stated. Effects on cell proliferation and cyclin D1 levels in the MCF-7-derived

cells, and STAT5 activity in CHO cells were confirmed with a preparation from the Goffin laboratory. Purified natural human PRL (NIDDK-PRL; lot AFP9042) was obtained through the National Hormone and Pituitary Program, and Dr. Parlow.

*PRL-deficient MCF-7 cell culture*

The MCF-7 derived subline (29) was grown in RPMI 1640 medium containing 10% horse serum and 50  $\mu$ M ganciclovir (GCV). The cells were transferred to the above medium minus the GCV four days prior to use. For growth assays, cells were plated at  $4 \times 10^5$  cells/60-mm tissue culture dish. After seeding, the cells were washed once with serum-free RPMI 1640 and cultured in serum-free RPMI 1640 for 48 h before treatment with vehicle, NIDDK-PRL, WT-PRL (unmodified PRL) or S179D-PRL (all at 100 ng/ml). The cells were harvested with trypsin at the indicated times and live cells counted using a hemocytometer.

*Western Analyses*

PRL-deficient MCF-7 cells were plated as described for the growth assays before treatments. Cells were harvested into 75  $\mu$ l lysis buffer (25 mM Tris (pH 8.0), 2 mM EDTA, 10% glycerol, 1% Triton X-100, 2 mM sodium orthovanadate, and 20 mM sodium fluoride). The cellular debris was removed by centrifugation at 10,000 rpm at 4° C for 10 min, and the protein concentration in the supernatant was determined using the bicinchoninic acid kit (Pierce Chemical Co., Rockford IL). Lysates (30  $\mu$ g protein) were electrophoresed through standard Laemmli SDS-polyacrylamide gels (12%), transferred to polyvinylidene fluoride membranes, and then probed with the appropriate antibodies. Membranes were blocked 4 h in 0.25% gelatin in 100 mM Tris-HCl (pH 7.5), 150 mM sodium chloride, and 0.1% Tween 20; washed once with 100 mM Tris-HCl (pH 7.5), 150

mM sodium chloride and 0.1% Tween 20; and incubated in primary antibody overnight at 4° C (cyclin D1, 1:500; STAT 5 pTyr<sup>694</sup>, 1:1000; STAT 5, 1:5000; Phospho-p44/42 ERK, 1:5000; p44/42 ERK, 1:1000; PRLR long form, 1:2000; PRLR intermediate form, 1:6250). Proteins were visualized using enhanced chemiluminescence as previously described (31). Quantification of the signals was performed using a Molecular Dynamics Personal SI densitometer and ImageQuant (version 4.2a) software (Molecular Dynamics, Inc., Sunnyvale, CA). For some experiments, the parental MCF-7 cell line and T47D line were similarly examined.

*CHO-K1 cell culture, transient transfection and reporter gene assays*

Chinese Hamster Ovary (CHO-K1) cells were maintained in Dulbecco's modified Eagle's medium/ F12 containing 5% FBS and penicillin/streptomycin (Gibco BRL Life Technologies, Gaithersburg, MD), and were transiently transfected using Superfect (Qiagen Inc., Valencia, CA) as described (30). The PRE3 plasmid consists of three copies of the consensus sequence for the STAT 5 binding site (TTCTTGGAA) from the  $\beta$ -casein promoter (prolactin response element, PRE), upstream of a luciferase reporter (29). The human long form PRLR construct was graciously provided by Dr. C. Clevenger (32), and the CMV- $\beta$ -galactosidase construct by Dr. C. Caskey (33).

Luciferase activity of cell lysates was determined as described (30). Luciferase values were corrected for transfection efficiency by determining the ratio of luciferase activity to  $\beta$ -galactosidase activity and expressed as relative luciferase units (RLU).

*Statistical Analyses*

Statistical analyses were performed using Prism v.3.02 (GraphPad Software, Inc., San Diego, CA).

## Results

### *S179D-PRL blocks WT-PRL-induced increase in numbers of PRL-deficient MCF-7 cells*

To determine if the different forms of PRL elicited different responses in our PRL-deficient mammary tumor cells, we evaluated the change in cell number following incubation with purified natural PRL (NIDDK-PRL; a mixture of unmodified and posttranslationally modified hormone) (22), PRL synthesized in *E. coli* and therefore without post-translational modifications (WT-PRL), or S179D-PRL similarly prepared (Fig. 1A). At 48 h, treatment with 100 ng/ml of either NIDDK-PRL or WT-PRL doubled the number of cells, whereas the same concentration of S179D-PRL had no effect. Preparations of S179D-PRL from both the Walker and Goffin laboratories yielded similar results. Furthermore, S179D-PRL was able to block the response to WT-PRL, with 10 ng/ml inhibiting WT-PRL (100 ng/ml) action about 50% (Fig. 1B). Since the PRL-deficient MCF-7 cells have a low rate of apoptosis under these conditions (unpublished observations), the higher number of cells that resulted from treatment with either NIDDK-PRL or WT-PRL primarily reflects an increase in proliferation.

### *S179D-PRL blocks WT-PRL-induced increase in cyclin D1*

Because of the key role of cyclin D1 in regulating the G<sub>1</sub>/S transition and mediating the proliferative response to PRL, we examined the effect of S179D-PRL on cyclin D1 levels. WT-PRL increased cyclin D1 levels in a dose dependent manner as we observed previously with NIDDK-PRL (29) (Fig. 2A). 100ng/ml WT-PRL yielded maximal stimulation, and was used for all subsequent studies. S179D-PRL had no detectable effect on cyclin D1 unless high concentrations (1,000 ng/ml), which only resulted in a modest change (Fig. 2B), were employed. Again, S179D-PRL was able to

compete with WT-PRL to decrease cyclin D1 to the level of the untreated control (Fig. 2C). To determine if S179D-PRL was able to inhibit PRL action in other mammary tumor cell lines, we examined the parental MCF-7 cells as well as T47D cells, both of which express endogenous PRL (29,34,35). As expected, WT-PRL only marginally increased cyclin D1 levels consistent with activity of the endogenous hormone, whereas S179D-PRL inhibited basal expression (Fig. 2D).

*S179D-PRL decreased WT-PRL-induced phosphorylation of STAT 5*

STAT 5 is a key mediator of PRL-induced increases in cyclin D1 transcription (30) as well as many other targets of PRL (36). Therefore we examined STAT 5 phosphorylation at Y694 after stimulation with WT-PRL or S179D-PRL using an antibody that recognizes this modification in both STATs 5a and 5b (Fig. 3A). WT-PRL elicits a robust response, but as for cyclin D1 levels, S179D-PRL displayed only very weak activity at the highest concentration (1,000 ng/ml). Tyrosine phosphorylation of STAT 5 in response to WT-PRL at this site was inhibited by increasing concentrations of S179D-PRL (Fig. 3B). However, the level of phosphorylation was never completely reduced to control levels even at 10,000 ng/ml (data not shown). To investigate the net effect on STAT 5 activity, a simple STAT 5 responsive enhancer linked to a reporter gene (PRE3-luciferase) was employed. The PRL-deficient MCF-7 cells exhibited only a weak activation (30%) of this promoter in response to PRL (data not shown). Therefore, we used Chinese hamster ovary (CHO) cells, which have been a useful model to examine PRL signaling, in order to quantitatively evaluate effects of S179D-PRL on STAT 5 activation. These cells produce negligible levels of PRL and only low levels of PRLR, so PRLR isoform complement can be controlled. In CHO cells co-transfected with the

human IPRLR and the strongly STAT 5-responsive PRE3-luciferase construct, WT-PRL increased luciferase expression 6-fold (Fig. 3C). S179D-PRL, prepared by both the Walker and Goffin laboratories, had no activity at 100 ng/ml, and only very low activity at 1,000 ng/ml, similar to endpoints in the MCF7-derived cells. When both WT-PRL and S179D-PRL were added together, S179D-PRL significantly but incompletely inhibited the WT-PRL-enhanced promoter activity (Fig. 3C), similar to the effect on phosphorylation of STAT 5 at Y694 in the MCF-7-derived cells.

*WT-PRL-stimulated ERK activation is attenuated by S179D-PRL in the MCF-7-derived cells*

PRL also utilizes other signaling pathways, including ERKs (1,3). WT-PRL stimulated ERK 1 and ERK 2 phosphorylation maximally at 15 min in this system (Fig. 4C). As was the case for STAT 5 phosphorylation, S179D-PRL demonstrated only slight activity toward ERKs at 1,000 ng/ml (Fig. 4A). In contrast to the ability of S179D-PRL to inhibit PRL-induced increases in cyclin D1 and STAT 5 phosphorylation, it was unable to decrease WT-PRL stimulated ERK 1 and ERK 2 phosphorylation at this time, even at concentrations of 10  $\mu$ g/ml (Fig. 4B). However, S179D-PRL shortened the duration of WT-PRL stimulated ERK phosphorylation (Fig. 4C). Whereas WT-PRL initiated a biphasic response, falling after a peak at 15 min and then rising slightly again at 135 min, equal concentrations of S179D-PRL brought levels to basal by 90 min and prevented the second rise.

*S179D-PRL increased IPRLR levels*

The PRLR gene is alternatively spliced to yield multiple receptor isoforms with different signaling capacities (for review, 3). Target cells express more than one isoform,

with relative and absolute levels varying with target and physiologic state. Exposure to ligand has been reported to augment or down regulate receptors in different experimental systems (for reviews, 37-39). MCF-7 cells express primarily the long PRLR isoform (iPRLR), although low levels of other isoforms are present (Brockman, unpublished observations). In the PRL-deficient MCF-7 cells, long term incubation (72h) with WT-PRL modestly reduced levels of the iPRLR (Fig. 5A,B). In marked contrast, S179D-PRL increased iPRLR levels by 4 fold over that time. Levels of the intermediate PRLR (iPRLR) isoform, which are present at much lower levels, did not change with treatment (Fig. 5A, lower blot). In the reciprocal of the experiments shown in Figs. 2C, 3B, and 4B, increasing concentrations of WT-PRL were employed to inhibit the effects of S179D-PRL. As shown in Fig. 5C, WT-PRL competes effectively for S179D-PRL signaling to this endpoint as well.

### Discussion

Mammary tumor cells, like many other extrapituitary cells, synthesize PRL (for review, 3,40). We know little about this PRL: the relatively low levels of expression compared to pituitary lactotrophs have limited our knowledge of post-translational modifications and how they may be altered by environmental context. Here we assessed the relative activities of unmodified hPRL and a molecular mimic of phosphorylated hPRL, S179D-PRL, in human mammary tumor cells in the absence of confounding endogenous PRL production. In this system, S179D-PRL was a weak PRL agonist for proliferation. However, it was an effective antagonist of unmodified PRL for this activity at low concentrations (0.4-4.0 nM) as measured by several indices, including net effects

on cell number, cyclin D1 levels, phosphorylation of STAT 5 at Y694, and the duration of ERK phosphorylation, which has been linked to cell cycle progression in other systems (41). Although S179D-PRL at 4nM reduced WT-PRL activity to unstimulated levels for some of these endpoints, including cell number and cyclin D1 levels, for others, including phosphorylation of Y694 of STAT5, acute ERK phosphorylation, and activation of a PRL-responsive enhancer, this antagonism was incomplete. Antagonistic activity was evident not only in this cell line, but also in the parental MCF-7 and T47D mammary tumor cell lines, where S179D-PRL reduced cyclin D1 levels below those of the unstimulated cells, arguably by opposing endogenously synthesized PRL.

These generally antagonistic activities of S179D-PRL at low concentrations observed in our MCF-7-derived cells contrast with more complex effects observed in other systems by both the Walker and Goffin laboratories. In rat Nb2 cells, which display high levels of a mutant form of the lPRLR with a partial deletion in the cytoplasmic domain (39), S179D-PRL effectively induced tyrosine phosphorylation of STAT 5a, and at least in the Walker laboratory, but not Goffin laboratory, also antagonized cell proliferation (23,24). In murine HC11 cells, S179D-PRL initiated only low levels of tyrosine phosphorylation but higher levels of serine phosphorylation of STAT 5a compared to unmodified hormone (27). However, S179D-PRL strongly activated ERKs in these cells, and selective inhibitors linked this pathway to the increase in  $\beta$ -casein transcripts. In contrast, in our studies using the MCF-7-derived cells, which express predominantly the lPRLR but also low levels of other PRLR isoforms, S179D-PRL did not inhibit the first phase of ERK activation, but did attenuate the second phase. Interestingly, Goffin and colleagues

observed signaling to ERKs after 15 min of exposure to S179D-PRL using conditions similar to those examined here in another human mammary tumor cell line, T47D (24). While differences in the hormone preparations from the Walker and Goffin laboratories have been cited as a reason for some of their apparently conflicting observations (24), this does not account for our findings in the MCF7 subline described here, since protein prepared in these laboratories had the same effects. Clearly, species differences in hormone and receptors (42), endogenous production of PRL, tissue origin and oncogenic mutations, as well as PRLR isoform complement, can confound results and will take some time to unravel. In the current study, we have eliminated the complication of endogenous PRL production and have used species appropriate hormone preparations.

The low concentrations of S179D-PRL necessary to exert antagonistic effects in the PRL-deficient MCF-7 cells as well as some studies of Nb2 cells (22) are consistent with a high affinity for the PRLR. However, Goffin and colleagues found that this mutant had about a 10-fold lower affinity for the human lPRLR stably overexpressed in 293 cells than WT-PRL, assessed by competition for binding with radiolabelled WT-hPRL (24). In the present studies in the PRL-deficient MCF-7 cells, similar concentrations of S179D-PRL were required to inhibit responses to WT-PRL, and of WT-PRL to block the S179D-PRL-induced increase in lPRLR levels. These data are consistent with the altered structure of this mutant modifying interactions with the PRLR, thereby affecting conformational changes in the receptor and consequently activation of downstream signaling pathways.

Factors governing total PRLR expression and relative levels of PRLR isoforms are not well understood (for review 37-39). Depending on the model system, concentration of PRL, and time course, PRL has been observed to both up-regulate and down-regulate membrane binding proteins. In contrast to downregulation of the iPRLR in the MCF-7-derived cells in response to WT-PRL, S179D-PRL treatment markedly increased iPRLR levels above those in cells cultured in serum-free media. These data suggest that at least some target cells may demonstrate increased sensitivity to WT-PRL signals mediated by this isoform following exposure to S179D-PRL. Levels of iPRLR, in contrast, were not affected by S179D-PRL; other PRLR isoforms (43,44) were not examined. Whether the S179D-PRL-induced increase in iPRLR represents reduced degradation and/ or increased synthesis was not ascertained in these studies. The interactions of S179D-PRL with the PRLR and the underlying mechanisms and trafficking of the PRLR isoforms subsequent to S179D-PRL binding are under investigation.

These studies demonstrate that low concentrations of S179D-PRL antagonize WT-PRL signaling to several pathways in breast cancer cells that may impact on carcinogenic behavior, particularly proliferation. These observations suggest that this compound may prove useful in understanding the actions of PRL in mammary tumorigenesis and progression, as well as in development of therapeutic approaches.

## FIGURE LEGENDS

**Fig. 1.** S179D-PRL inhibits WT-PRL-induced growth of PRL-deficient cells. A. Cells were plated at equal densities, cultured in serum-free medium for 48 h, and then treated with the appropriate ligand (NIDDK-PRL, WT-PRL or S179D-PRL, all at 100 ng/ml). The number of live cells was counted at each time point as described in *Materials and Methods*. Results are expressed as the mean  $\pm$  SE of triplicate plates. B. Cells were plated at equal densities, cultured in serum-free medium for 48 h, and then treated with vehicle, WT-PRL (100 ng/ml) alone or WT-PRL (100 ng/ml) + S179D-PRL (1 ng/ml, 10 ng/ml or 100 ng/ml). The number of live cells was counted 48 h after treatment. The number of cells in the vehicle control was subtracted from the experimental plates and the increases in response to hormone treatments are shown. Both A and B are representative experiments of at least three experiments. Different lower case letters indicate significant differences among treatments using ANOVA followed by Student-Newman-Keuls post test ( $P<0.05$ ).

**Fig. 2.** S179D-PRL blocks WT-PRL induction of cyclin D1 protein. A,B,C. After 48 h in serum free media, PRL-deficient MCF-7 cells were treated for 6 h with vehicle or hormones as shown and cyclin D1 levels in lysates determined by Western analysis. Blots shown are representative of at least 3 experiments. D. Representative Western analysis of cyclin D1 in lysates from parental MCF 7 and T47D cells at 6 h after treatment.

**Fig. 3.** S179D-PRL blocks WT-PRL-induced phosphorylation of STAT5 at Y694 in PRL-deficient MCF-7 cells, but does not block PRE-driven luciferase activity in transfected CHO cells.

A,B. After 48 h in serum free media, PRL-deficient MCF-7 cells were treated for 15 min with vehicle or hormones as shown and levels of STAT 5 phosphorylated Y694 or total STAT 5 in lysates determined by Western analysis using antibodies that recognize both STATs 5a and 5b under these conditions. Blots shown are representative of at least 3 experiments. C. CHO cells were transiently transfected with the PRE3-luciferase reporter construct, IPRLR, and  $\beta$ -galactosidase constructs as described in Materials and Methods, and treated as indicated with WT-PRL, S179D-PRL, or a combination of the two forms, using preparations from the Walker (solid bars) and Goffin (open bars) laboratories. After 24 h, samples were assayed for luciferase activity and  $\beta$ -galactosidase activity was used to correct for transfection efficiency. Activity is presented relative to untreated PRE3-transfected cells and data represent the mean of five separate experiments, +/- SEM. Different letters indicate significant differences between treatment groups (Students t test,  $p < 0.05$ ).

**Fig. 4.** S179D-PRL inhibits the second phase of ERK phosphorylation by WT-PRL.

A,B. After 48 h in serum free media, PRL-deficient MCF-7 cells were treated for 15 min with vehicle or hormones as shown and phospho-ERK or ERK levels in lysates determined by Western analysis. Blots shown are representative of at least 3 experiments. C. After 48 h in serum free media, cells were treated for the indicated

times with vehicle or hormones as shown and phospho-ERK and ERK levels in lysates determined by Western analysis.

**Fig. 5.** S179D-PRL increases levels of lPRLR in PRL-deficient MCF-7 cells. Cells were cultured for 24 h in serum-free media, treated with vehicle or hormone as shown, and levels of PRLR determined by Western analyses. A. Representative Western analysis of lPRLR and iPRLR following hormone treatment (100 ng/ml) at 0 and 72 h after treatment as shown. B. Change in lPRLR levels compared with control (0 h) from three independent experiments (mean  $\pm$  SE), vehicle (black bars), WT-PRL (gray bar) and S179D-PRL (white bar). Different letters indicate significant differences among treatments using ANOVA followed by Student-Newman-Keuls post test ( $P < 0.05$ ). C. Representative Western analysis of lPRLR at 72 h after treatment. Blot is representative of at least 3 experiments.

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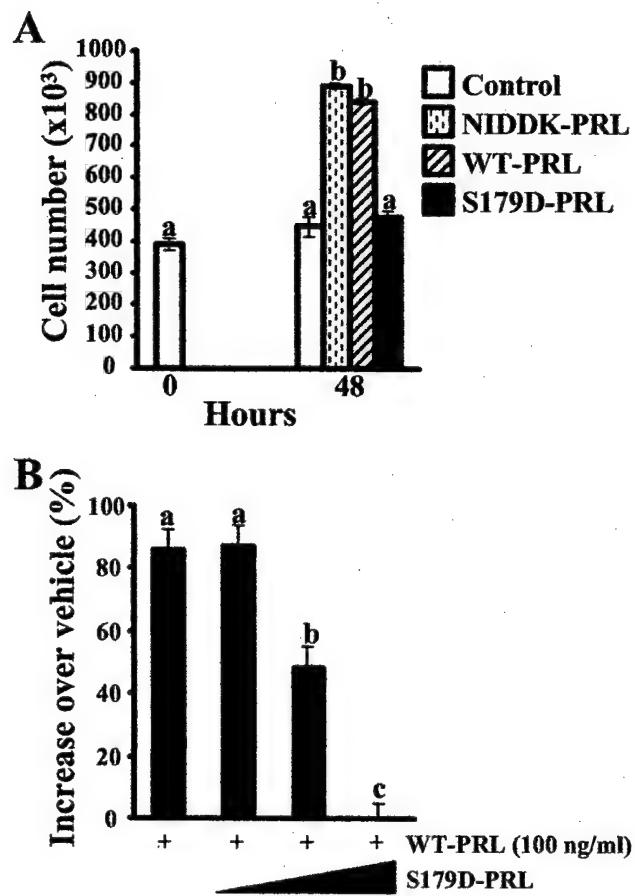
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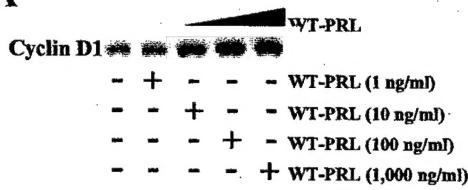
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Figure 1

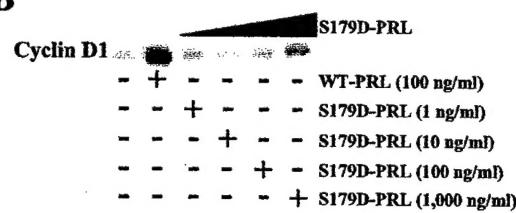


## Figure 2

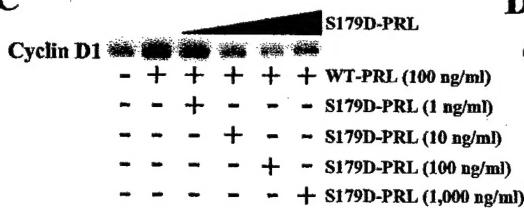
**A**



**B**



**C**



**D**

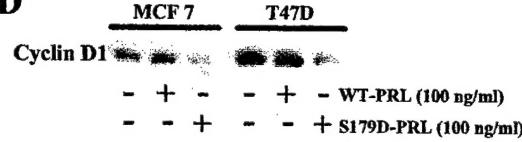
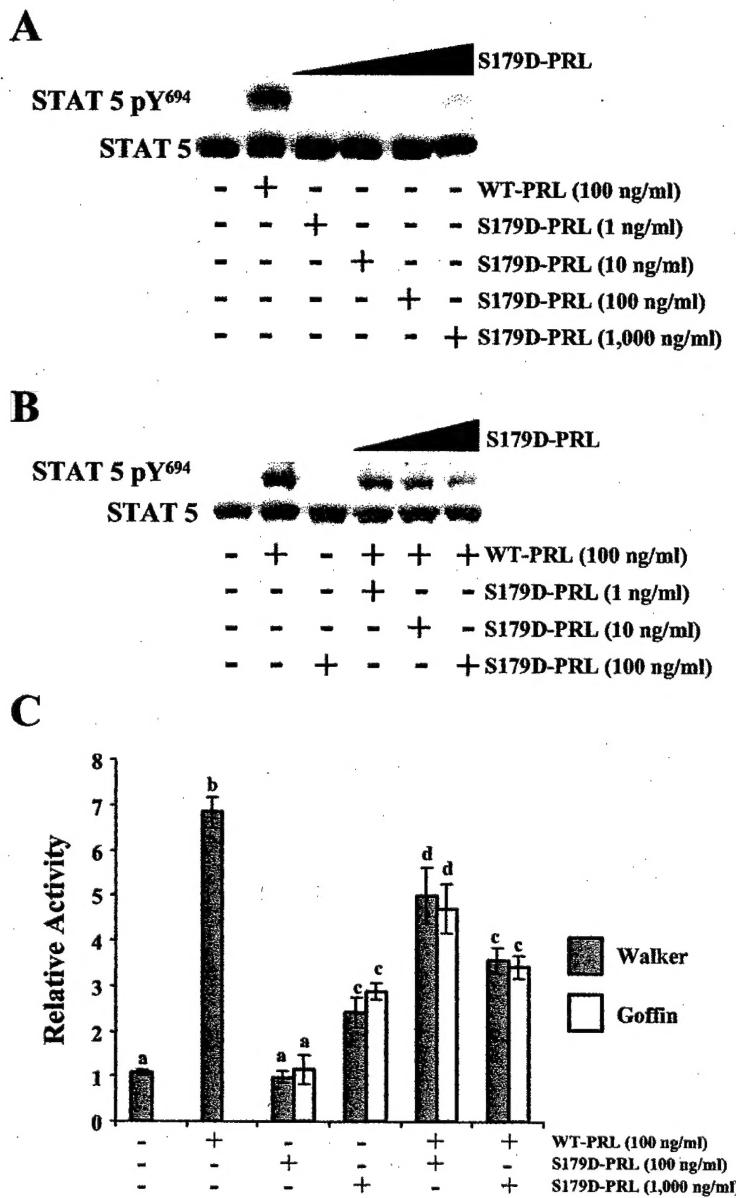
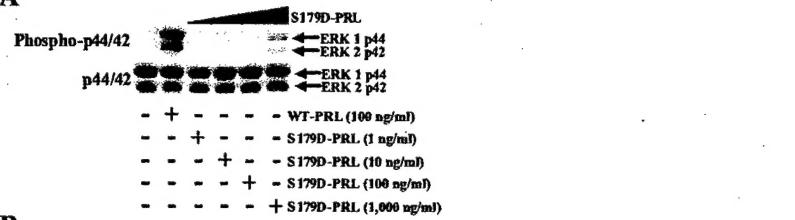


Figure 3

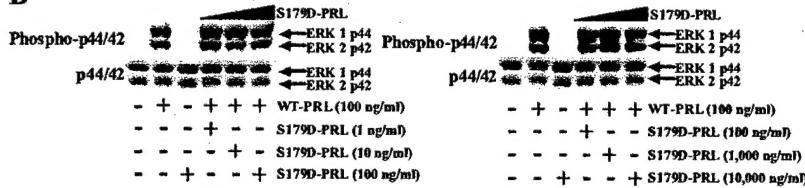


**Figure 4**

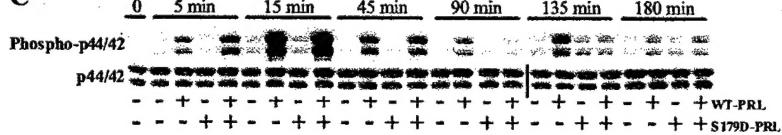
**A**



**B**

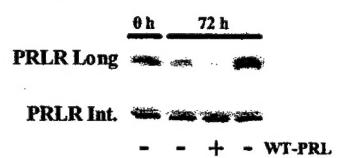


**C**

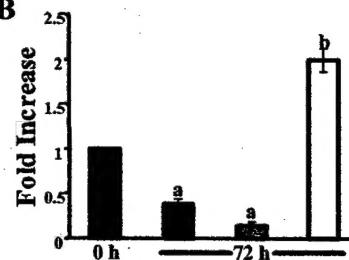


**Figure 5**

**A**



**B**



**C**

